Analyse der Interferon-induzierten Acetylierung des Transkriptionsfaktors STAT1 und Übertragung des Acetylierungsmotivs auf STAT3 und monomeres STAT1

Dissertation

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von Diplom Biochemiker
Torsten Ginter
geboren am 07.04.1982 in Hoyerswerda

1. Gutachter: PD Dr. Oliver H. Krämer (Jena)
2. Gutachter: Prof. Dr. Karlheinz Friedrich (Jena)
3. Gutachter: Prof. Dr. Friedemann Horn (Leipzig)

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2. Einleitung

2.1 STAT-Proteine

Die Entdeckung der ersten signal transducer and activator of transcription (STATs) gelang vor rund 20 Jahren durch die Arbeitsgruppe von J. Darnell [Shuai, 1992]. Seitdem hat die Wissenschaft viel über den zugrundeliegenden Signalweg und die verschiedenen Funktionen der STAT-Proteine in Erfahrung gebracht. STATs regulieren vornehmlich Gene des Immunsystems, aber sie spielen auch eine entscheidende Rolle in der Krebsentstehung und bei Entzündungsprozessen. Eine präzise Abstimmung der Aktivierung und Deaktivierung dieser Transkriptionsfaktoren ist Voraussetzung für die Aufrechterhaltung der Gesundheit (Homöostase). Auf welche Weise dieses Gleichgewicht gewährleistet wird, ist eine faszinierende wissenschaftliche Frage.

2.1.1 Aufbau und Struktur der STAT-Proteine

Aus dem menschlichen Organismus wurden sieben STATs isoliert, die sich vermutlich evolutionär aus einem gemeinsamen Vorfahren entwickelt haben. Dies wird begründet durch deren chromosomale Verteilung und durch homologe STATs in Modellorganismen [Schindler, 2008]. *Drosophila* besitzt beispielsweise nur ein STAT-Protein [Miyoshi, 2001].
Strukturell sind alle STATs aus sieben Proteindomänen aufgebaut (Abbildung 1). Die N-terminale Domäne (NTD) ist gut konserviert und wird für Dimerbildung von unphosphorylierten STATs benötigt [Braunstein, 2003, Mao, 2005, Zhong, 2005, Mertens, 2006]. Daran schließt sich die Coiled-coil Domäne (CC) an, welche aus einem 4-Helix-Bündel-Motiv besteht und Interaktionen mit regulatorischen Proteinen vermittelt, sowie an Kernimport- /Exportprozessen beteiligt ist [McBride, 2003, Schindler, 2007]. Die DNA-Bindedomäne (DBD) ist zwischen den verschiedenen STATs ebenfalls gut konserviert und ermöglicht eine robuste Bindung aller STATs an palindromische DNA-Erkennungssequenzen im Promotor ihrer Zielgene [Schindler, 2008]. Weiterhin ist bekannt, dass die DBD an der Vermittlung des Kernimports und Exports beteiligt ist [Becker, 1998, McBride, 2003]. Die folgende Linker-Domäne (LD) trägt ebenfalls zur DNA-Bindung bei und ist einbezogen in einen kontinuierlichen basalen Kernexportprozess [Bhattacharya, 2003] der STAT-Proteine. Die sich anschließende src-homology-2 (SH2) Domäne ist für alle STATs hoch konserviert und unverzichtbar für die Bildung aktiver Phosphotyrosin-vermittelter Dimere [Shuai, 1994]. Des Weiteren ermöglicht sie die Bindung der STATs an den Zytokinrezeptor [Schindler, 2007]. Die kleinste Domäne ist das Phosphotyrosin-Motiv,

welches für jedes STAT ein Tyrosin aufweist [Lim, 2006]. Das Motiv ist in der Raumstruktur nach außen gerichtet, was eine Phosphorylierung des Tyrosins erlaubt. In dieser phosphorylierten Form können STAT-Dimere über eine SH2-Interaktion gebildet werden [Mertens, 2006]. Am C-Terminus befindet sich die Transaktivierungsdomäne (TAD). Sie ist in ihrer Sequenz und Länge variabel, aber beinhaltet bei vielen STATs konservierte Serin-Phosphorylierungsstellen, die für eine Interaktion mit verschiedenen Coaktivatoren benötigt werden [Lim, 2006]. Zusätzlich spielt die TAD eine zentrale Rolle im Ubiquitin-vermittelten Proteinabbau der STATs [Wang, 2000, Tanaka, 2005].

Abbildung 1:Schematische Darstellung der STAT-Proteindomänen
NTD - N-terminale Domäne, CC – coiled-coil Domäne, DBD - DNA-Bindedomäne, LD - Linker-Domäne, SH2 - SH2-Domäne, Y -Phosphotyrosin-Motiv, TAD - Transaktivierungsdomäne

2.1.2 Funktionen der verschiedenen STATs

STAT1 und STAT2 welche 1992 als erste Vertreter der STAT-Familie gefunden wurden [Stark, 2012], sind dafür bekannt im angeborenen Immunsystem Interferon (IFN) Typ I- und IFN Typ II-vermittelte Reaktionen zu steuern (siehe 2.1.5). Diese Signalwege sind von großer Bedeutung für die antivirale und antibakterielle Abwehr [Chapgier, 2006, Takaoka, 2006]. STAT1 wirkt pro-inflammatorisch, anti-proliferativ und unterstützt meist die Apoptose [Chin, 1996, Stephanou, 2000, Hong, 2002, Schroder, 2004, Krämer, 2006]. Jedoch wurde in manchen Arbeiten auch eine anti-apoptotische Wirkung von STAT1 nachgewiesen [Kovacic, 2006, Stronach, 2011]. Oft wirkt STAT3 auf gewisse Weise den Funktionen von STAT1 entgegen und gilt als Onkogen [Bromberg, 1999, Regis, 2008]. STAT3 treibt die Expression von anti-apoptotischen und proliferativen Genen an und fördert die Tumorinvasion [Dechow, 2004, Azare, 2007, Zugowski, 2011]. STAT3 vermittelt weiterhin über Interleukin 10 (IL10) anti-inflammatorische Prozesse und ist für die Bildung von regulatorischen T_H17 T-Zellen verantwortlich [Wang, 2004, Kortylewski, 2005, Regis, 2008]. Diese Prozesse können die immunologische Abstoßung von entarteten Zellen einschränken. STAT3 kann aber auch im Zusammenspiel mit dem nuclear factor κB (NF-κB) Entzündungsprozesse regulieren, die die Krebsentstehung fördern [Yu, 2009]. Die Notwendigkeit von STAT3 für die embryonale Entwicklung belegt die Letalität von STAT3 *knock out* Mäusen [Takeda, 1997]. STAT4 steuert spezifisch die IL12-abhängige Entwicklung von naiven $CD4^+$ T-Zellen zu IFNγ-sekretierenden T_H1 T-Zellen und zusätzlich die Aktivierung von IFNγ-sekretierenden NK T-Zellen [Thierfelder, 1996]. Auch an der Generierung von T_H17 T-Zellen aus $CD4^+$ T-Zellen ist STAT4 beteiligt [Mathur, 2007]. STAT5a und STAT5b gehen aus einer Genduplikation hervor und spielen eine bedeutende

Rolle in der Bildung von Erythrozyten, lymphoiden Zellen und bei der Erhaltung der hämatopoetischen Stammzellpopulation [Yao, 2006, Wang, 2009]. Beide STAT5 Isoformen werden großteils durch dieselben Zytokine aktiviert, jedoch favorisiert Prolaktin STAT5a und Wachstumshormon STAT5b [Schindler, 2008]. Weiter ist bekannt, dass die Aktivierung von STAT5 in verschiedenen Krebsarten fehlreguliert ist und dass STAT5 als Tumorpromotor gilt [Huang, 2002].
STAT6 wird durch IL4 und IL13 aktiviert und reguliert die Entwicklung von T_H2 T-Zellen, B-Zell Funktionen sowie die Aktivität von Mastzellen [Kaplan, 1996, Wurster, 2000]. In manchen Leukämien und Lymphomen ist STAT6 konstitutiv aktiviert [Bruns, 2006].

2.1.3 Aktivierung der STAT-Proteine

STATs sind zytoplasmatisch lokalisierte Transkriptionsfaktoren, die über verschiedenste Liganden aktiviert werden und daraufhin in den Zellkern translozieren [Schindler, 2007].
Im klassischen Aktivierungsweg binden Zytokine oder Wachstumsfaktoren extrazellulär an die Transmembranrezeptoruntereinheiten, was eine Zusammenlagerung mehrerer Untereinheiten (meist zwei) zum Rezeptorkomplex zur Folge hat (Abbildung 2) [Gough, 2008]. Eine dadurch induzierte Konformationsänderung des intrazellulären Rezeptorteils bewirkt eine Aktivierung von assoziierten Janus Kinasen (JAKs) und Tyrosinkinasen (TYK) durch Transphosphorylierung. Die JAKs wiederum phosphorylieren den intrazellulären Rezeptorteil an bestimmten Tyrosinresten. Diese Phosphotyrosine werden durch vorgeformte, STAT-Dimere über SH2-Interaktionen gebunden [Kretzschmar, 2004, Mertens, 2006].
Nach einer NTD-vermittelten Konformationsänderung (siehe 2.1.4) binden die STATs wechselseitig über Phosphotyrosin-SH2-Interaktionen. Das nun aktivierte STAT-Dimer translozíert über einen importin-vermittelten Transportmechanismus in den Zellkern, wo es Zielgene induzieren kann. Die Promotoren der Zielgene enthalten bestimmte palindromische Erkennungssequenzen woran das parallele Dimer in einer „Nussknacker"-ähnlichen Struktur bevorzugt bindet [Lim, 2006]. Interessanterweise induziert die DNA-Bindung eine zusätzliche Serinphosphorylierung, welche für eine gesteigerte Transkriptionsaktivität verantwortlich ist [Sadzak, 2008].

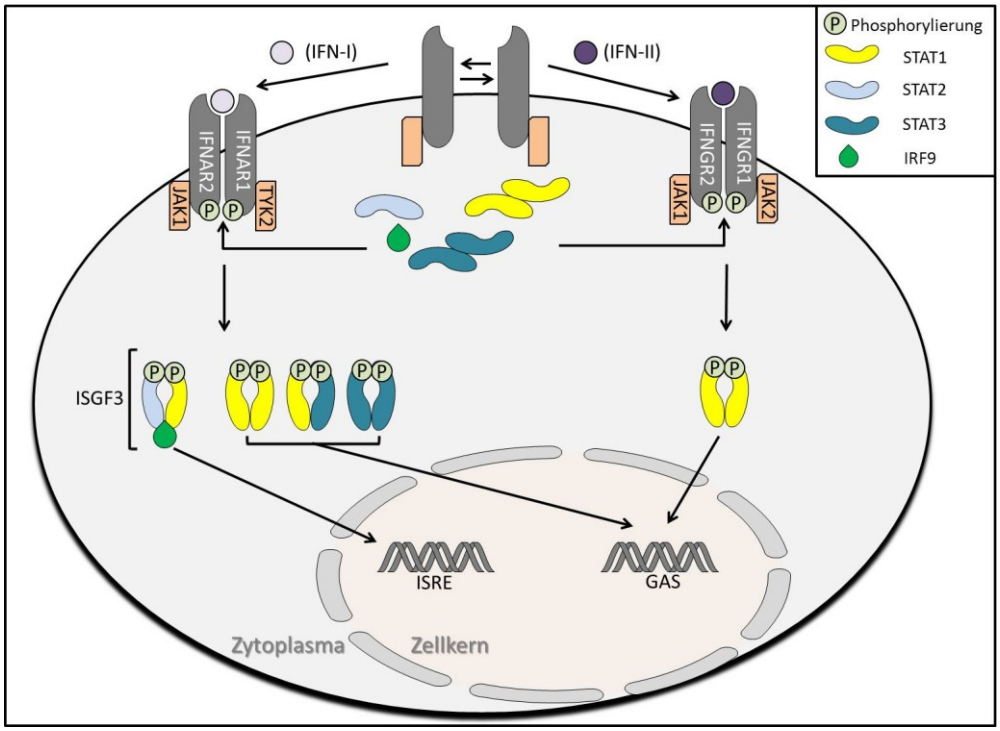

Abbildung 2: **Interferon Typ I/II induzierte Aktivierung des JAK-STAT Signalwegs**
Abhängig vom Liganden werden verschiedene Rezeptoren gebunden und unterschiedliche STATs aktiviert. Typ I Interferone (IFN-I) führen zur Bildung des interferon stimulated gene factor 3 (ISGF3), welches an interferon stimulated response elements (ISRE) bindet. Zusätzlich können STAT1/STAT1, STAT1/STAT3 und STAT3/STAT3 Dimere gebildet werden, die an Interferon-γ activated sites (GAS) binden. Typ II Interferon (IFN-II) bewirkt die Assoziierung von STAT1-Homodimeren, die GAS-Sequenzen erkennen. IFNα-Rezeptoruntereinheit 1/-2 (IFNAR1/-2); IFNγ-Rezeptoruntereinheit 1/-2 (IFNGR1/-2); Phosphorylierung (P); Janus Kinase 1/-2 (JAK); Tyrosinkinase 2 (TYK2); Legende oben rechts.

Abhängig vom Liganden und von der Zusammensetzung des STAT-Dimers werden unterschiedliche Erkennungssequenzen gebunden. Alle STATs, bis auf STAT2, können an Interferon-γ activated sites (GAS) oder GAS-ähnliche (z.B. SIE - sis-inducible element und APRE - acutephase response element) Erkennungssequenzen binden [Decker, 1991, Schindler, 2008]. Heterotrimere aus STAT1, STAT2 und interferon regulatory factor 9 (IRF9) werden auch als interferon stimulated gene factor 3 (ISGF3-Komplex) bezeichnet und binden an interferon stimulated response element (ISRE) Erkennungssequenzen (Abbildung 2) [Reich, 1989, Reich, 2007]. Die Transkription der Zielgene wird durch Rekrutierung verschiedener Coaktivatoren und durch RNA-Polymerase II vorangetrieben [Kornberg, 1999].

2.1.4 Die funktionelle Rolle der NTD

Die NTD entwickelte sich evolutionär später und ist in STATs von *Drosophila melanogaster*, *Danio rerio* und *Vertebraten*, jedoch nicht in *Dictyostelium discoideum* und *Caenorhabdites elegans* zu finden [Kawata, 1997, Wang, 2006]. Evolutionsbiologen vermuten, dass die

Einführung der NTD den STATs neue Regulationsmöglichkeiten erlaubte, die insbesondere für die Flexibilität der DNA-Bindung wichtig sind [Wang, 2012].

STATs liegen in der Zelle als vorgeformte antiparallele Dimere vor. Die antiparallele Konformation ist durch NTD-Interaktionen vermittelt und erleichtert die anschließende Phosphorylierung der STATs durch Exposition der betreffenden Tyrosine. Danach findet eine Konformationsänderung zum parallelen STAT-Dimer statt, welches wechselseitig über Phosphotyrosin/SH2-Domänen-Interaktion stabilisiert ist [Mertens, 2006]. Die Inaktivierung der STAT-Dimere nach der DNA-Bindung wird ebenfalls durch eine Reorientierung zum antiparallelen Dimer eingeleitet, welche eine erleichterte Dephosphorylierung des Tyrosins erlaubt [Zhong, 2005] (Abbildung 3).

Weiterhin können NTD-Interaktionen zusätzlich eine Tetramerisierung beziehungsweise sogar Oligomerisierung von STATs gewährleisten. Solche NTD-vermittelten Formierungen von verschiedenen Dimeren zu Oligomeren auf DNA-Ebene ermöglichen eine Feinabstimmung der Genexpression. Gerade Promotoren mit unterschiedlichen STAT-Bindungsstellen können so präzise reguliert werden. Bislang wurden NTD-vermittelte Tetramere von STAT1, STAT3, STAT4 und STAT5 auf verschiedenen Promotoren nachgewiesen [Timofeeva, 2012b].

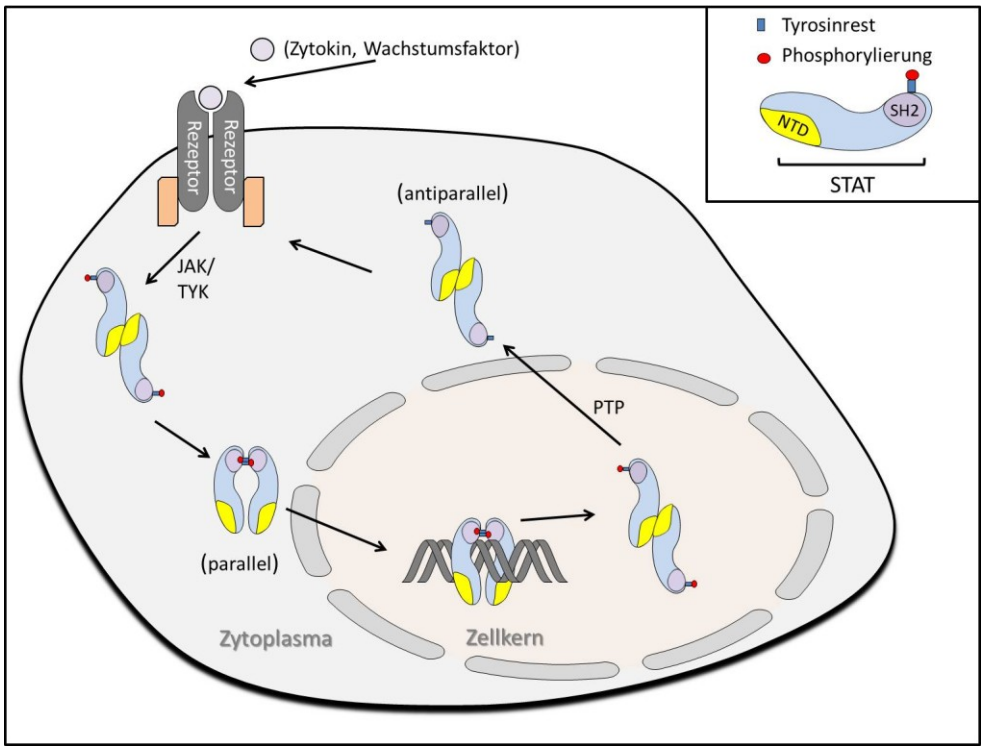

Abbildung 3: Konformationsänderung der STATs im Aktivierungs-/Inaktivierungszyklus
Im inaktiven Zustand liegen STATs als vorgeformte antiparallele Dimere vor. Der nach außen gerichtete Tyrosinrest erleichtert die Phosphorylierung durch rezeptorassoziierte Kinasen (JAK/ TYK). Anschließend findet eine Konformationsänderung zum parallelen Dimer statt, welches über wechselseitige Phosphotyrosin-SH2-Interaktion stabilisiert wird. Dieses parallele Dimer bindet im Zellkern an DNA und aktiviert die Zielgenexpression. Eine Reorientierung zum antiparallelen Dimer erlaubt die Dephosphorylierung durch Protein-Tyrosin-Phosphatasen (PTP), welche zur Inaktivierung des Dimers führt. Das antiparallele Dimer liegt letztlich wieder im Zytoplasma für einen erneuten Zyklus vor. Janus Kinase (JAK); Tyrosinkinase (TYK); Legende oben rechts.

Bei der Dimerbildung von unphosphorylierten STATs (U-STATs) scheint die NTD ebenfalls einen Einfluss zu haben. G. Starks Labor wies solche U-STATs, welche auch Einfluss auf die Zielgenexpression nehmen, für STAT1 und STAT3 nach [Yang, 2008a]. Aufgrund der NTD-vermitteln Interaktionen liegt ein Großteil der Dimere in der antiparallelen Konformation vor und nur ein kleinerer Teil besitzt parallel orientierte Dimere [Timofeeva, 2012b].

2.1.5 Interferone

Die 1957 durch Isaacs und Lindenmann entdeckten Interferone (IFNs) gehören zu den Vier-Helix-Bündel Zytokinen und haben vielfältige Aufgaben im angeboren Immunsystem [Isaacs, 1957, Borden, 2007]. Sie werden in drei Klassen unterteilt.

Die Typ I IFN Klasse umfasst acht Subtypen wovon die bekanntesten IFNα/-β sind. IFNα/-β können von allen Zellen nach Virusinfektion produziert und sekretiert werden. Alle Typ I IFNs vermitteln ihre Aktivität über einen IFNα-Rezeptor (IFNAR)-Komplex, welcher aus den Untereinheiten IFNAR1 und IFNAR2 besteht und die Bildung eines STAT1/STAT2/IRF9 Heterotrimers ermöglicht (Abbildung 2). Dieses Heterotrimer induziert zahlreiche antivirale Gene [Gale, 2005, Takaoka, 2006].

Die Typ II IFN Klasse weist nur IFNγ auf, welches ebenfalls antivirale Prozesse vermittelt, aber nur von aktivierten T-Zellen und NK-Zellen sekretiert werden kann [Takaoka, 2006]. Es nutzt einen IFNγ-Rezeptor (IFNGR)-Komplex, der aus zwei Untereinheiten besteht (IFNGR1 und IFNGR2). IFNγ führt klassisch zu einer Bildung von STAT1 Homodimeren (Abbildung 2) [Schindler, 2008].

Das Typ III IFN, IFNλ, wurde erst 2003 von zwei unabhängigen Forschergruppen entdeckt und besteht beim Menschen aus drei IFNλ Subtypen [Kotenko, 2003, Sheppard, 2003, Vilcek, 2003]. Es zeigt homologe Eigenschaften zu Typ I IFN. So wird es ebenfalls nach Virusinfektion ausgeschüttet und bewirkt die Bildung von ISGF3 mit anschließender Aktivierung gleicher Zielgene. Jedoch besitzt Typ III IFN eine andere Struktur und benötigt die spezifischen Rezeptoruntereinheiten IFNλR1 oder IL28Rα und IL10R2. Unbekannt sind noch die Rezeptor-assoziierten Kinasen und weitere Forschungen sind ebenfalls nötig um die nachgeschalteten Signalwege aufzuklären [Takaoka, 2006].

Einzelne Zytokine können oft unterschiedliche STATs aktivieren und führen zur Bildung verschiedener Homo- und Heterodimere. Für IFNα ist beispielsweise bekannt, dass es in verschiedenem Ausmaß alle STAT-Proteine aktivieren kann [Schindler, 2008]. Klassisch werden hauptsächlich STAT1 und STAT2 phosphoryliert und bilden mit IRF9 den ISGF3-Komplex (Abbildung 2). Jedoch sind zelltypabhängig auch Assoziierungen von STAT1/STAT3 Heterodimeren sowie STAT1, STAT3, STAT4 und STAT5 Homodimeren möglich [Wesoly, 2007]. Wie die Zelle die richtige Wahl trifft ist nicht vollständig geklärt.

Bekannt ist, dass unterschiedliche Mengen von Coaktivatoren und Corepressoren sowie die Konzentration eines Zytokins einen Einfluss auf die Entscheidung haben können welche Dimere gebildet werden [Regis, 2008, Icardi, 2012].

2.1.6 Inaktivierung der STAT-Proteine

Eine strikt regulierte Inaktivierung der STATs ist ebenso unverzichtbar wie die schnell vermittelte Aktivierung. Ist dieses Gleichgewicht gestört, können verschiedene Krankheitsbilder resultieren. Aufgrund der hauptsächlichen Funktionen der STATs im adaptiven und angeboren Immunsystem sowie bei Entzündungen sind dies meist Autoimmunerkrankungen, aber auch die Krebsentstehung wird durch dauerhaft aktiviertes STAT3 und STAT5 gefördert [Klampfer, 2006, Zugowski, 2011, O'Shea, 2012].

Die Phosphorylierung eines Tyrosins ist bei allen STATs eine Voraussetzung für eine volle Aktivierung. Demnach ist es nicht verwunderlich, dass Phosphatasen eine wichtige Rolle bei der Inaktivierung der STATs spielen. SHP-1, SHP-2 (SH2-containing PTP 1/-2) und TCP45 (T cell protein tyrosine phosphatase) katalysieren die Dephosphorylierung der STATs im Zellkern, was entscheidend für den nukleären Export ist [Tenev, 2000, McBride, 2003, Schindler, 2007]. Bekannt sind aber auch Phosphatasen, die den Rezeptor und die JAKs dephosphorylieren [Kisseleva, 2002, Mustelin, 2005].

Eine weitere Möglichkeit der Deaktivierung sind SOCS-Proteine (suppressor of cytokine signaling). Diese Proteine sind Zielgene der STATs selbst und werden in einer negativen Rückkoppelungsschleife sehr schnell nach Stimulation exprimiert. SOCS-Proteine inhibieren direkt JAKs durch SH2-vermittelte Bindung eines Phosphotyrosins in der Aktivierungsschleife, welche für die volle Aktivität der Kinase autophosphoryliert sein muss [Yasukawa, 1999]. Des Weiteren ist bekannt, dass sie über die SH2-Domäne auch intrazelluläre Phosphorylierungsstellen des Rezeptors, die als STAT-Bindungstellen fungieren, blockieren können [Qing, 2005, Fenner, 2006]. Zusätzlich können SOCS-Proteine einen E3-Ubiquitin-Ligase-Komplex rekrutieren und darüber zum proteasomalen Proteinabbau von SOCS-Bindungspartnern (z.B. JAKs) beitragen [Croker, 2008].

PIAS-Proteine (protein inhibitor of activated STAT) sind im Gegensatz zu SOCS-Proteinen konstitutiv exprimiert. Sie interagieren direkt mit phosphorylierten STATs und verhindern deren Bindung an DNA, wodurch die Zielgenexpression gehemmt wird [Liu, 2004]. Interessanterweise sind PIAS-Proteine auch small ubiquitin-like modifier (SUMO)-E3-Ligasen und für STAT1 konnte eine Sumoylierung durch PIAS1 beschrieben werden [Kotaja, 2002, Ungureanu, 2003], ob die Sumoylierung selbst für die Kontrolle von STAT1 relevant ist, ist jedoch offen [Rogers, 2003].

2.2 Acetylierung als Post-translationale Modifikation (PTM)

PTMs können die Eigenschaften von Proteinen entscheidend verändern. Sie koordinieren Aktivierung/Inaktivierung, entscheiden über die Lokalisation, ermöglichen Interaktionen oder verhindern diese und sie regulieren die Proteinstabilität. Die Acetylierung wurde erstmals als Modifikation von Histonen entdeckt, was die Namensgebung der beteiligten Enzyme beeinflusste (siehe 2.2.1). Bis heute sind hunderte Nicht-Histon-Proteine, die acetyliert werden können bekannt. Darunter sind auch viele Transkriptionsfaktoren, wie beispielsweise p53, NF-κB oder STAT-Proteine [Spange, 2009].

2.2.1 Histonacetyltransferasen (HATs) und Histondeacetylasen (HDACs)

Das Gleichgewicht zwischen Acetylierung und Deacetylierung ist sehr dynamisch und wird durch zwei gegensätzliche Enzymklassen reguliert. HATs katalysieren die Übertragung von Acetylgruppen von Acetyl-CoA auf die ε-NH_2-Gruppe bestimmter Lysinreste. Die Deacetylasen benötigen für die Entfernung der Acetylierung bestimmte Cofaktoren und werden darum in Zn^{2+}-abhängige HDACs und NAD^+ -abhängige SIRTs (sirtuine) unterteilt [Spange, 2009]. Die HDACs werden auf Basis ihrer Vertreter in *S. cerevisiae* in Klasse I (HDAC 1, 2, 3, 8), Klasse II (HDAC 4, 5, 6, 7, 9, 10) und Klasse IV (HDAC 11) eingeordnet. Klasse III umfasst SIRT 1-7 [Yang, 2008b, Delcuve, 2012]. Im Gegensatz zu HDACs variieren HATs stärker in Struktur und Funktion, wodurch eine Klassifizierung erschwert wird [Spange, 2009]. Fehlerhafte Regulation und anormale Proteinlevel von HATs und HDACs sind für verschiedene Krebsarten beschrieben [Sakuma, 2006, Wilson, 2006]. Aufgrund dessen werden verstärkt Inhibitoren für diese Enzyme entwickelt. Für HDACs existieren bereits vielversprechende Inhibitoren (HDACi), die in späten klinischen Phasen oder sogar für Anwendungen im Menschen zugelassen sind [Prince, 2009, Spange, 2009, Müller, 2010, Giannini, 2012].

2.2.2 Regulation der STATs durch Acetylierung

Neben der Tyrosin-Phosphorylierung, welche eine beträchtliche Bedeutung für die STAT-Aktivierung hat (siehe 2.1.3), stellt die Acetylierung eine weitere bedeutende PTM dar. Für jedes der sieben STATs ist eine Acetylierung beschrieben (Tabelle 1) und soll hier chronologisch aufgeführt werden.

Für STAT6 wurde als erstes eine Acetylierung entdeckt, welche positiv auf die Expression von Zielgenen wirkt [Shankaranarayanan, 2001]. Die genaue Acetylierungsstelle konnte in dieser 2001 veröffentlichten Arbeit nicht identifiziert werden. Laut

massenspektrometrischen (MS) Analysen gibt es mehrere acetylierte Lysinreste in STAT6 [phosphosite].

Zwei unabhängige Gruppen fanden 2005 zeitgleich eine Acetylierung des C-terminalen Lysins an Position 685 in STAT3 [Wang, 2005, Yuan, 2005]. Beide nutzten Punktmutanten des vermeintlichen Lysins, um die Auswirkungen der Acetylierung zu analysieren. Eine Mutation von Lysin zu Glutamin (K→Q) simuliert, aufgrund der strukturellen Ähnlichkeit zu Acetyllysin, eine konstitutive Acetylierung. Der Austausch zum Arginin (K→R) verhindert Acetylierung, begründet durch die Mesomerie-stabilisierte Guanidine-Gruppe der Seitenkette [Krämer, 2010]. Wang et al. und Yuan et al. beobachteten für die K685R Mutante eine verminderte DNA-Bindung, eine gestörte Dimerisierung und eine beeinträchtigte Zielgenexpression. Weitere Acetylierungsstellen wurden in der Nachbarschaft von Lysin 685 und im N-terminalen Bereich gefunden [Ray, 2005, Nadiminty, 2006, Ray, 2008, Lee, 2009, Nie, 2009]. Alle Publikationen bis auf Gupta et al. beschreiben eine positive Auswirkung der STAT3-Acetylierung auf den Signalweg [Gupta, 2011]. Die Gruppe um Gupta wies in B-Zell Lymphomen eine reduzierte STAT3-Tyrosinphosphorylierung und damit einhergehend eine verminderte Transkriptionsaktivität nach.

Zahlreiche Publikationen existieren, die eine Regulation von STAT1-vermittelten Signalwegen durch HDACs nahe legen. Unter Verwendung von HDACi oder durch HDAC negative Zellen und siRNA gegen HDACs konnte ein hemmender Effekt von Acetylierung auf die STAT1 vermittelte Zielgeneexpression gezeigt werden [Genin, 2003, Klampfer, 2003, Nusinzon, 2003, Chang, 2004]. Direkte STAT1-Acetylierung konnte erstmals 2006 durch unsere Arbeitsgruppe belegt werden [Krämer, 2006]. Danach wurde diese Beobachtung mehrmals bestätigt [Guo, 2007, Hayashi, 2007, Tang, 2007, Cudejko, 2011, Stronach, 2011, Banik, 2012]. Wir konnten in Folgearbeiten zeigen, dass der inhibitorische Effekt der STAT1-Acetylierung auf einer verstärkten Dephosphorylierung beruht [Krämer, 2009, Ginter, 2012]. Zusätzlich wurden die von uns postulierten Acetylierungsstellen K410 und K413 zusammen mit anderen Stellen in MS-Analysen bestätigt [phosphosite, Wieczorek, 2012].

Die Bindung von IFNα an den IFNAR bewirkt eine Acetylierung seines zytoplasmatischen Teils durch die HAT CBP (CREB binding protein). CBP acetyliert im Folgenden auch STAT2, STAT1 und IRF9, die Komponenten des ISGF3-Komplexes [Tang, 2007]. Die Acetylierung von K390 in der STAT2 DBD ist entscheidend für die Bildung des Heterotrimers und für die Aktivierung der antiviralen Genregulation [Tang, 2007]. Tang et al. und Datenbanken (Tabelle 1) listen noch zusätzliche Positionen auf, die vermutlich acetyliert werden.

Tabelle 1: Acetylierungsstellen der STAT-Proteine

STAT Protein	Vermutete Acetylierungsstelle	HAT	HDAC	Stimulus	Quelle
STAT1	K410, K413	CBP	HDAC1,-2,-3,-4	IFNα, IFNγ HDACi Cisplatin	[phosphosite, Krämer, 2006, Guo, 2007, Hayashi, 2007, Tang, 2007, Krämer, 2009, Cudejko, 2011, Stronach, 2011, Ginter, 2012]
	K679	?	?	?	[Ma, 2010]
	K173	?	?	?	[phosphosite]
STAT2	K390, K182, K184, K194, K197, K384, K415, K419, K592	CBP, p300	?	IFNα	[Tang, 2007]
	K158, K384	?	?	?	[phosphosite]
	K375	?	?	?	[phosida, phosphosite, Choudhary, 2009]
STAT3	K685	CBP, p300	HDAC1,-2,-3 SIRT1	OSM, IFNα	[Wang, 2005, Yuan, 2005, Nadiminty, 2006, Nie, 2009, Lee, 2012]
	K49, K87	p300	HDAC1	IL6	[Ray, 2005, Hou, 2008, Ray, 2008]
	K679, K707, K709	CBP, p300	SIRT1	IL6, OSM, Diät, SIRTi/SIRT Aktivator	[Nie, 2009]
STAT4	K691	?	?	?	[Ma, 2010]
STAT5a	K84, K384	?	?	?	[phosphosite]
	K696	p300	?	IL7/FLT3L	[phosida, Van Nguyen, 2012]
STAT5b	K701	?	?	SAHA, MS275	[phosida, Choudhary, 2009]
	K359, K694, K701	CBP, p300, GCN5, PCAF	?	Prolactin IL7/FLT3L	[Ma, 2010, Van Nguyen, 2012]
	K84, K384	?	?	?	[phosphosite]
STAT6	?	CBP, p300	?	IL4	[Shankaranarayanan, 2001]
	K636	?	?	?	[Ma, 2010]
	K129*, K619*, K621*	?	?	?	[phosphosite]

Aufgeführt sind alle bekannten Acetylierungspositionen und die Acetylierung induzierenden Liganden sowie die betreffenden HATs und HDACs. OSM – Oncostatin M; SIRTi – SIRT inhibitor; IL – Interleukin; FLT3L – fms-like tyrosine kinase receptor-3 ligand; GCN5 - for general control nonderepressible 5; PCAF - p300/CBP-associated factor; SAHA (Vorinostat), MS275 (Etinostat); ? – unbekannt; aus [Wieczorek, 2012].

Ma et al. haben mit Hilfe von MS-Analysen und Positions-spezifischen Acetylierungsantikörpern für STAT5b die Acetyllysinreste K359, K694 und K701 nachgewiesen. Zudem wurde ein positiver Zusammenhang zwischen Acetylierung und Dimerisierung von STAT5 beobachtet [Ma, 2010]. Die Gruppe um Ma berichtete auch von einer Acetylierung des C-terminalen Lysinrests in STAT1 (K679) und STAT4 (K691). Für STAT4 ist dies bislang die einzige beschriebene Acetylierungsposition.

In einer kürzlich erschienenen Publikation wurde für STAT5 ein erstaunlicher Crosstalk zwischen Acetylierung und Sumoylierung aufgedeckt. Van Nguyen et al. entdeckten in SENP1 (SUMO-specific protease 1) negativen Mäusen (SENP1-/-) eine gestörte Differenzierung der T- und B-Lymphozyten sowie eine verringerte STAT5 Zielgenexpression [Van Nguyen, 2012]. SENP1 ist eine Protease, die Sumoylierungen von Lysinresten entfernt und folglich konnte in SENP1-/- Zellen eine verstärkte Sumoylierung von STAT5 beobachtet werden. Interessanterweise konkurriert diese inaktivierende Sumoylierung mit der Acetylierung der Lysinreste 696 (STAT5a) und 701 (STAT5b) [Van Nguyen, 2012]. In der genannten Arbeit wurde zudem gezeigt, dass die Phosphorylierung von STAT5 eine Voraussetzung für die anschließende Sumoylierung ist, vermutlich da sie eine Translokation in den Nukleus erlaubt. Auf welche Weise die Acetylierung mit der Phosphorylierung zusammenhängt ist nicht vollständig geklärt. Für STAT5a ist dazu nichts bekannt, aber für STAT5b ist die Phosphorylierung von Y699 keine Bedingung für die Acetylierung von K694 und K701 [Ma, 2010]. Van Nguyen et al. postulieren einen Aktivierungs-/Inaktivierungszyklus für STAT5, welcher durch die Abfolge von verschiedenen PTMs reguliert wird (Abbildung 4). Aktives phosphoryliertes und acetyliertes STAT5-Dimer transloziert in den Nukleus und wird dort deacetyliert und dephosphoryliert. Eine SUMO-E3-Ligase modifiziert betreffende Lysinreste und gewährleistet damit ein inaktives STAT5-Dimer. SENP1 entfernt anschließend diese Sumoylierung und STAT5 steht für eine neue Aktivierungsrunde zur Verfügung [Van Nguyen, 2012].

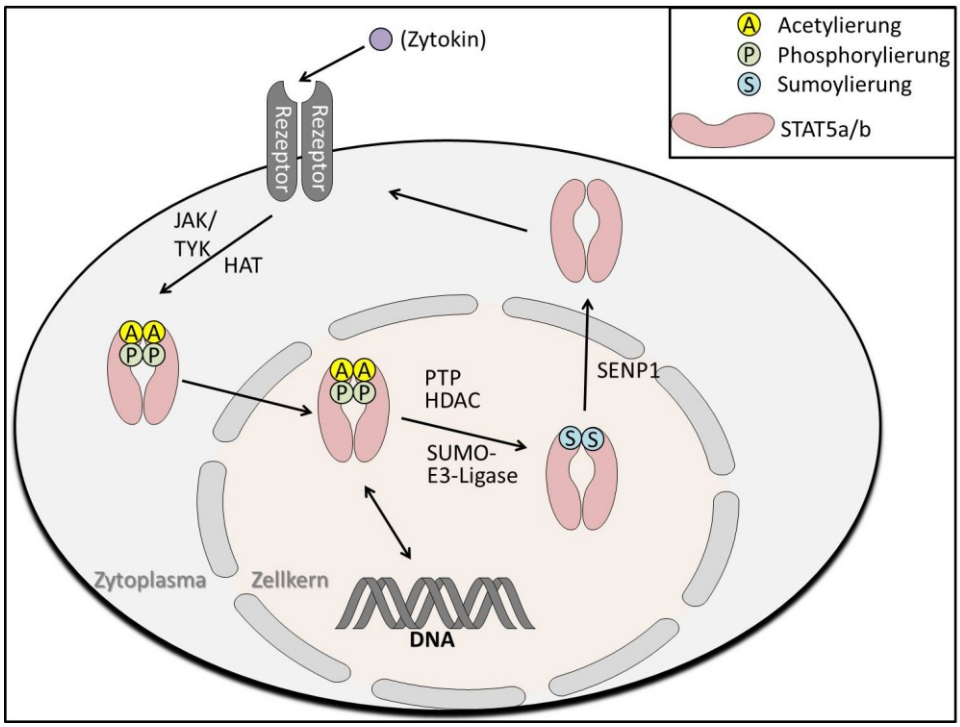

Abbildung 4: Regulation von STAT5 durch die Abfolge verschiedener PTMs
Über eine Zytokin induzierte Rezeptoraktivierung werden STAT5-Dimere durch rezeptorassoziierte Kinasen (JAK und TYK) phosphoryliert. Histonacetyltransferasen (HAT) vermitteln die Acetylierung von bestimmten Lysinresten in STAT5a/b. Die zeitliche Reihenfolge zwischen Phosphorylierung und Acetylierung ist unklar. Beide PTMs erlauben die Translokation in den Zellkern, wo STAT5 Zielgene transkribiert werden. Die Inaktivierung von STAT5-Dimeren geschieht über Dephosphorylierung durch Protein-Tyrosin-Phosphatasen (PTP) und Deacetylierung durch Histondeacetylasen (HDAC). Zusätzlich katalysiert eine SUMO-E3-Ligase die Sumoylierung des zuvor acetylierten Lysinrests. Die Protease SENP1 entfernt die inaktivierende Sumoylierung des STAT5-Dimers und erlaubt seine Translokation zurück in das Zytoplasma, wodurch STAT5 für einen neuen Aktivierungszyklus zur Verfügung steht. SUMO-specific protease 1 (SENP1); small ubiquitin-like modifier (SUMO); Janus Kinase (JAK); Tyrosinkinase (TYK); Legende oben rechts.

Die molekularen Mechanismen, die die Substratspezifität von bestimmten HDACs und HATs für einzelne STATs festlegen, sind bislang ungeklärt. Zudem ist es interessant in wie weit andere PTMs oder gar konkurrierende Lysinmodifikationen (z.B. Ubiquitinylierung, Methylierung, Sumoylierung) mit Acetylierung wechselwirken. Entsprechende Beispiele sind bereits für STAT1 und STAT5 bekannt (siehe 2.2.2 und Abbildung 4) [Krämer, 2009, Van Nguyen, 2012].

2.2.3 STAT1 und STAT3 – Zwei Homologe mit ungleichen Eigenschaften

STAT1 und STAT3 sind strukturell sehr ähnlich, weisen jedoch oft entgegengesetzte Funktionen auf [Melen, 2001, Regis, 2008]. STAT1 wirkt häufig anti-proliferativ, pro-apoptotisch und pro-inflammatorisch. STAT3 reguliert diese Prozesse meist gegenläufig (siehe 2.1.2). Interessanterweise wirkt sich auch eine Acetylierung in der Regel umgekehrt auf STAT1 und STAT3 aus (siehe 2.2.2).

Unsere Gruppe konnte für STAT1 an den Positionen 410 und 413 eine IFNα induzierte Acetylierung nachweisen [Krämer, 2006]. Weiterhin konnten wir zeigen, dass diese Modifikation einen hemmenden Effekt auf von STAT1 vermittelte Signalprozesse hat. Dieser negative Einfluss beruht auf einem Acetylierungs/Phosphorylierungs-Umschaltprozess [Krämer, 2009]. Phosphoryliertes STAT1 wird von CBP acetyliert und kann anschließend besser durch die Phosphatase TCP45 gebunden werden. Die resultierende Dephosphorylierung fördert den Kernexport und stellt STAT1 nach Deacetylierung durch HDAC3 für einen weiteren Aktivierungszyklus zur Verfügung [Krämer, 2009].

Bemerkenswerterweise sind die entsprechenden Lysine 410 und 413, die sich in der DBD befinden, für alle STAT-Proteine bis auf STAT2 und STAT3 erhalten [Melen, 2001]. STAT2 besitzt an diesen Stellen ein Valin- und ein Argininrest, STAT3 sogar zwei Argininreste, die eine Acetylierung ausschließen. Wie in 2.2.2 beschrieben unterliegt STAT3 N-terminalen und C-terminalen Acetylierungen, die aktivierend wirken. Die C-terminale Position ist auch für STAT1 konserviert. Die N-terminale jedoch nur teilweise (Abbildung 5).

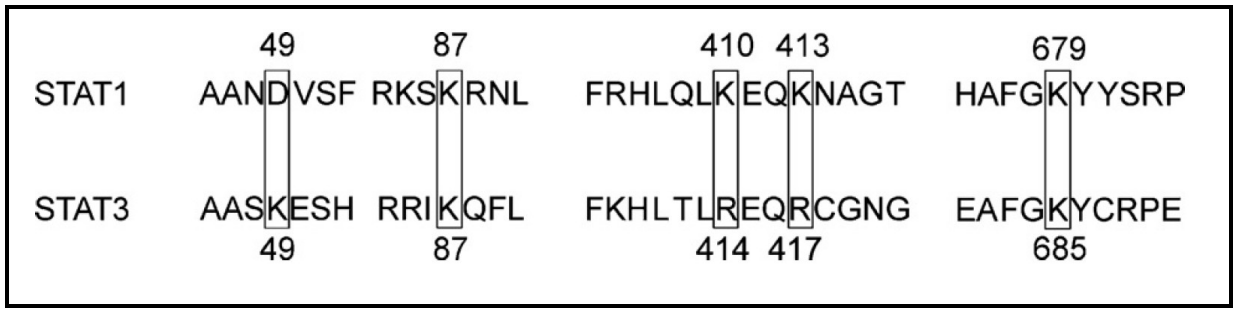

Abbildung 5: Aminosäuresequenzvergleich von STAT1 und STAT3
STAT1 und STAT3 weisen eine hohe Homologie auf, jedoch sind die N-terminalen Lysine (49, 87) nur teilweise konserviert. In der DBD ersetzen Arginine in STAT3 die entsprechenden Lysine in STAT1. Die C-terminale Position ist in beiden erhalten; nach [Krämer, 2010].

Die Lysin- beziehungsweise Argininreste in der DBD scheinen für STAT1 und STAT3 evolutionär hoch konserviert zu sein. Dies zeigt ein Sequenzvergleich über verschiedene Arten hinweg. Selbst phylogenetisch vom Menschen weit entfernte Spezies wie *Danio rerio* und *Xenopus laevis* (Krallenfrosch) zeigen die entsprechenden Aminosäuren [Wieczorek, 2012]. In Anbetracht der durch HDACi induzierten Inaktivierung von STAT1 und der gegensätzlichen Aktivierung von STAT3, lässt sich eine evolutionär etablierte Regelung der Funktionen beider STATs durch Acetylierung vermuten. Dies könnte erklären warum die strukturell sehr ähnlichen STATs, STAT1 und STAT3, entgegengesetzte Funktionen ausüben.

3. Übersicht zu den Manuskripten

Manuskript 1

Status: bei Zeitschrift veröffentlicht am 7. März 2012

Titel: Histone deacetylase inhibitors block IFNγ-induced STAT1 phosphorylation.

Autoren: Torsten Ginter, Carolin Bier, Shirley K. Knauer, Kalsoom Sughra, Dagmar Hildebrand, Tobias Münz, Theresa Liebe, Regine Heller, Andreas Henke, Roland H. Stauber, Werner Reichardt, Johannes A. Schmid, Katharina F. Kubatzky, Thorsten Heinzel, Oliver H. Krämer,

Zeitschrift: Cellular Signalling

Inhaltsangabe: IFNγ und HDACi bewirken eine Acetylierung von STAT1, die zu einer stärkeren Interaktion mit TCP45 führt. Dadurch wird STAT1 schneller dephosphoryliert und somit inaktiviert. Acetylierung simulierende STAT1-Glutamin-Mutanten binden vermindert DNA und zeigen eine gestörte Transkriptionsaktivität, können jedoch durch einen funktionellen STAT-Heterodimerpartner teilweise in ihren Funktionen wiederhergestellt werden.

Eigenanteil: Alle Abbildungen bis auf Abbildung 1C, 2D, 3C-D, 6E und Anhang S3, S4, S5, S7 beruhen auf Versuchen, die durch mich durchgeführt wurden. Daten für Abbildung 6F wurden in Zusammenarbeit mit Heike Urban (AG Henke) generiert, wobei sie die Infektion und kolorimetrische Auswertung der Zellen übernahm. Konzeption und Inhalt des Textes wurde von mir in Zusammenarbeit mit Oliver Krämer ausgearbeitet. Das Layout der Abbildungen wurde von mir in Abstimmung mit Oliver Krämer geplant und ausgeführt.

Manuskript 2

Status: bei Zeitschrift veröffentlicht am 12. Juli 2012

Titel: Acetylation modulates the STAT signaling code

Autoren: Martin Wieczorek[1], Torsten Ginter[1], Peter Brand[1], Thorsten Heinzel, Oliver H. Krämer

[1] Diese Autoren haben gleiche Anteile zu der Publikation beigetragen.

Zeitschrift: Cytokine & Growth Factor Reviews

Inhaltsangabe: STAT-Proteine bestimmen eine Vielzahl von zellulären Prozessen und werden durch PTMs entscheidend reguliert. Die Acetylierung hat sich in den letzten Jahren als eine entscheidende PTM des JAK-STAT Signalwegs herausgestellt. Dieser Artikel gibt eine Übersicht über bislang bekannte Acetylierungsstellen und deren Auswirkung für nachgeschaltete Signalprozesse sowie beleuchtet evolutionäre Aspekte der STAT-Acetylierung.

Eigenanteil: Ich habe wie die Co-Autoren Martin Wieczorek und Peter Brand maßgeblichen Anteil an der Planung und der Ausführung des Manuskriptes gehabt. Mein Schwerpunkt lag dabei auf den Teilstücken, die sich mit STAT1 und STAT3 befassen. Weiterhin habe ich Datenrecherche und die Konzeption von Tabelle 1 und 2 sowie Abbildung 3 übernommen.

Manuskript 3

Status: Buchkapitel online seit Januar 2013

Titel des Kapitels: Acetylation of Endogenous STAT Proteins

Autoren: Torsten Ginter, Thorsten Heinzel, Oliver H. Krämer

Buch: *JAK-STAT Signalling:* Methods and Protocols

Reihe: Methods in Molecular Biology

Verlag: Springer Science + Business Media New York

Inhaltsangabe: Acetylierung als PTM ist im JAK-STAT Signalweg weit verbreitet und wurde für mehrere STAT-Proteine beschrieben. Dennoch ist der Nachweis der Acetylierung nicht trivial und der Experimentator muss zahlreiche Vorgaben einhalten, um diese Modifikation erfolgreich detektieren zu können. Dieses Buchkapitel befasst sich, am Beispiel der STAT1-Acetylierung, mit den nötigen Rahmenbedingungen, die zum Nachweis der Acetylierung erforderlich sind.

Eigenanteil: Das gesamte Manuskript, bis auf Abbildung 1 und 2, wurde von mir in Abstimmung mit Oliver Krämer und Thorsten Heinzel geplant und ausgearbeitet. Für Abbildung 1 und 2 wurden die experimentellen Daten von Oliver Krämer zur Verfügung gestellt.

Manuskript 4

Status*:* Manuskript in Vorbereitung; geplante Veröffentlichung in *Cellular and Molecular Life Sciences*

Titel: Regulation of STAT3 and STAT1 by acetylation-phosphorylation cassettes

Autoren: Torsten Ginter, Christian Kosan, Carolin Bier, Roland H. Stauber, Andreas Henke, Thorsten Heinzel, and Oliver H. Krämer

Inhaltsangabe: STAT1-vermittelte Signale werden durch Acetylierung bestimmter Lysine in der STAT1-DBD, welche eine verstärkte Dephosphorylierung bewirken, gehemmt. Eine Übertragung von diesem Acetylierungsmodul auf STAT3 bewirkt eine Inaktivierung des vormals durch C-terminale Acetylierung aktivierbaren STAT3. Ebenso können überaktive STAT1-NTD-Mutanten, die unfähig sind NTD-vermittelte Dimere zu bilden, durch Einführung von Acetylierungs-simulierenden Glutaminen deaktiviert werden.

Eigenanteil: Text, Abbildungen und Gliederung des Manuskripts wurden in Zusammenarbeit mit Oliver Krämer entworfen. Alle zugrunde liegenden Versuche bis auf

jene für Abbildung 1C und 4B habe ich ausgeführt. Die Daten für Abbildung 5B wurden in Zusammenarbeit mit Heike Urban (AG Henke) generiert. Sie übernahm die Infektion und kolorimetrische Auswertung der Zellen.

4. Manuskripte

Manuskript 1: Histone deacetylase inhibitors block IFNγ-induced STAT1 phosphorylation

Cellular Signalling 24 (2012) 1453–1460

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Histone deacetylase inhibitors block IFNγ-induced STAT1 phosphorylation

Torsten Ginter [a], Carolin Bier [b], Shirley K. Knauer [c], Kalsoom Sughra [d], Dagmar Hildebrand [e], Tobias Münz [a], Theresa Liebe [a], Regine Heller [f], Andreas Henke [g], Roland H. Stauber [b], Werner Reichardt [h], Johannes A. Schmid [d], Katharina F. Kubatzky [e], Thorsten Heinzel [a], Oliver H. Krämer [a,*]

[a] Center for Molecular Biomedicine (CMB), Dept. of Biochemistry, University of Jena, Germany
[b] Dept. of Molecular and Cellular Oncology, University Hospital, Mainz, Germany
[c] Centre for Medical Biotechnology, Dept. of Molecular Biology II, University of Duisburg–Essen, Germany
[d] Center for Physiology and Pharmacology, Dept. of Vascular Biology, Medical University, Vienna, Austria
[e] Dept. of Infectious Diseases, Medical Microbiology and Hygiene, University Hospital Heidelberg, Germany
[f] CMB, Dept. of Molecular Cell Biology, Jena University Hospital, Jena, Germany
[g] Dept. of Virology and Antiviral Therapy, Jena University Hospital, Jena, Germany
[h] Dept. of Medical Technology and Biotechnology, University of Applied Sciences, Jena, Germany

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ABSTRACT

Signal transducer and activator of transcription 1 (STAT1) is important for innate and adaptive immunity. Histone deacetylase inhibitors (HDACi) antagonize unbalanced immune functions causing chronic inflammation and cancer. Phosphorylation and acetylation regulate STAT1 and different IFNs induce phosphorylated STAT1 homo-/heterodimers, e.g. IFNα activates several STATs whereas IFNγ only induces phosphorylated STAT1 homodimers. In transformed cells HDACi trigger STAT1 acetylation linked to dephosphorylation by the phosphatase TCP45. It is unclear whether acetylation differentially affects STAT1 activated by IFNα or IFNγ, and if cellular responses to both cytokines depend on a phosphatase-dependent inactivation of acetylated STAT1. Here, we report that HDACi counteract IFN-induced phosphorylation of a critical tyrosine residue in the STAT1 C-terminus in primary cells and hematopoietic cells. STAT1 mutants mimicking a functionally inactive DNA binding domain (DBD) reveal that the number of acetylation-mimicking sites in STAT1 determines whether STAT1 is recruited to response elements after stimulation with IFNγ. Furthermore, we show that IFNα-induced STAT1 heterodimers carrying STAT1 molecules mimicking acetylation bind cognate DNA and provide innate anti-viral immunity. IFNγ-induced acetylated STAT1 homodimers are though inactive, suggesting that heterodimerization and complex formation can rescue STAT1 lacking a functional DBD. Apparently, the type of cytokine determines how acetylation affects the nuclear entry and DNA binding of STAT1. Our data contribute to a better understanding of STAT1 regulation by acetylation.

1. Introduction

Interferons are cytokines that regulate the expression of genes determining cellular fate and anti-viral defense. IFNα and IFNβ belong to class I IFNs and IFNγ is classified as a class II IFN. Class III IFNs are cytokines with biological properties similar to type I IFNs [1]. IFNs bind to their cognate receptors. Subsequently, Janus kinases (JAKs) phosphorylate the receptor as well as a specific set of STAT proteins on tyrosine residues [2,3]. Phosphorylated STAT homo-/heterodimers enter the nucleus where they induce the expression of target genes [4–6]. In response to type I IFNs, several STATs are activated, and a STAT1–STAT2–IRF9 (ISGF3) complex is formed to activate transcription from IFN-stimulated response sites (ISRE). In contrast, IFNγ specifically induces phosphorylation of STAT1 at tyrosine 701 (Y701; abbreviated as p-STAT1). Phosphorylated STAT1 homodimers promote transcription from IFNγ-activated sites (GAS) [2,3]. Upon DNA binding, STAT1 also becomes serine phosphorylated to induce transcription [7]. Although phosphorylation crucially regulates many functions of STAT1, unphosphorylated STAT1 already associates with other STAT molecules in uninduced cells [5,8,9]. Of note, such preformed dimers control STAT-dependent gene expression [10,11].

To prevent excessive cytokine stimulation, IFN responses have to be regulated precisely. Extensive spatial and structural reorientation of the STAT dimer, generating a parallel to antiparallel conformational transition, permits STAT1 dephosphorylation by the phosphatase TCP45 [12]. Homodimerization of STAT1 via its N-terminal domain (NTD) allows this structural transition for presentation of pY701, which would otherwise be buried within the SH2 domain of another STAT partner molecule.

* Corresponding author at: Center for Molecular Biomedicine (CMB), Dept. of Biochemistry, University of Jena, Hans-Knöll-Str. 2, D-07745, Germany. Tel.: +49 3641 949362; fax: +49 3641 949352.
E-mail address: Oliver.Kraemer@uni-jena.de (O.H. Krämer).

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In addition to phosphorylation, STAT1 can be acetylated. Phosphorylation and signaling by STAT1 upon exposure to IFNs, lipopolysaccharide, alloantigenic activation, and the chemotherapeutic cisplatin are counteracted by STAT1 acetylation. At present, most data agree with STAT1 acetylation and with the observation that acetylation results in a switch to inactivate STAT1 [5,8,10,13–23].

A STAT1 mutant carrying lysine (K) to glutamine (Q) exchanges at positions 410/413 mimics the inactive acetylated STAT1. This $STAT1^{K410Q,K413Q}$ (abbreviated as $STAT1^{QQ}$ in the following text) allows testing how acetylation affects STAT1. $STAT1^{QQ}$ has functions reminiscent of STAT1 acetylated in response to previous IFN stimulation and after treatment of cells with HDACi [5,10]; e.g. a lack of tyrosine phosphorylation and transcriptional activation capacity. Consistent with the outstanding role of TCP45 for STAT-dependent signaling [5,12,24], attenuation of this phosphatase (PTP) by vanadate or more specifically by RNAi restores phosphorylation and signaling of acetylated wild-type STAT1 and of $STAT1^{QQ}$. Furthermore, a substrate trapping TCP45 binds acetylated STAT1 [5], which suggests that TCP45 has a high affinity for acetylated STAT1. HDACi prevent the removal of acetyl groups from lysine residues by histone deacetylases (HDACs), increase STAT1 acetylation and inhibit STAT1 signaling [25]. Of note, HDACi counteract aberrant and excessive immune reactions. It is believed that the inhibition of STAT1 signaling contributes to anti-inflammatory effects of HDACi [25]. Evidence for such a regulatory mechanism in primary non-transformed human cells has not been provided yet. It has equally not been resolved whether STAT1 acetylation requires phosphorylation and nuclear translocation. Moreover, STAT1 homo- versus heterodimers have physiologically relevant different functions [2,3,7], but it is unclear if STAT1 dimerization with other STATs determines the functional outcome of STAT1 acetylation.

In order to elucidate how acetylation affects phosphorylation of STAT1 homodimers, we treated primary and transformed cells with IFNγ to specifically induce phosphorylation of STAT1 homodimers. We show that HDACi block phosphorylation of STAT1 by IFNγ. Moreover, phosphorylated STAT3 can rescue the inert state of acetylated STAT1, i.e. STAT1 lacking an intact DBD can be functionally rescued when other STATs are also tyrosine phosphorylated. Our data further reveal that the number of "motifs" which mimic acetylated residues in the STAT1 DBD controls STAT1 phosphorylation and activity. These findings suggest that IFNα and IFNγ can generate diversity within a context including acetylated STAT1.

2. Material and methods

2.1. Cell lines, transfections, microscopy

Cells were maintained, treated and transfected as described [10]. Whereas transfection of excessive amounts of STAT1 causes cell death and outcompetes TCP45, stable expression of STAT1 in U3A cells was achieved comparable to its endogenous level in parental 2fTGH cells [5]. We analyzed mutant STAT1 molecules in U3A cells as they are a genetically defined cellular model lacking STAT1. HUVECs were cultured in M199 medium containing 15% fetal calf serum, 5% human serum, and 7.5 μg/ml endothelial mitogen [26]. Cells were characterized by flow cytometry staining for platelet endothelial cell adhesion molecule-1 (>98% PECAM-1 positive). Experiments were carried out with the first or second passage. Generation of primary bone marrow cells (BMCs) and bone marrow-derived dendritic cells (BMDCs) is described in the Supplementary materials. Immunofluorescence staining for STAT1-GFP was done as described [10]. Cells were incubated with 10^3 U/ml IFNα, 50 ng/ml IFNγ, 50 ng/ml IL6, 10 ng/ml leptomycin B (LMB) or 0.1–1 μM trichostatin A (TSA). Vanadate was used as noted in [5,10].

2.2. FRET microscopy and analysis

Normalized, corrected FRET-values (NFRET [27]) were derived with the PixFRET-plugin of ImageJ and mean intensities were determined for regions of interest. Further details can be found in the Supplementary materials.

2.3. Plasmids

Human STAT1α constructs were described before [10]. The RFP-TCP45 expression construct was made by cloning human TCP45 into pHcRed1 (Clontech). A reported mutation of pcDNA3.1 TOPO $STAT1^{K410,413Q}$ at position 636 [23] could be excluded by DNA sequencing (GATC-Biotech, Germany) (Supplemental Fig. S1).

2.4. Luciferase reporter assays

Luciferase reporter assays were performed as in [5]. Data shown are representative for independently repeated experiments.

2.5. Intracellular FACS staining and cell cycle analysis

For the analysis of p-STAT1 and STAT1, cells were treated with valproic acid (VPA, 2 mM) or MS-275 (5 μM) for 24 h or left unstimulated. Then cells were treated with INFγ (100 ng/ml) for 30 min. After washing with PBS cells were fixed with 4% paraformaldehyde/PBS for 20 min at room temperature. Subsequently, cells were permeabilized in 0.1% Triton X-100/PBS for 5 min at room temperature. Afterward cells were blocked in 10% FCS/PBS for 15 min at room temperature and then incubated with 1:50 p(Y701) STAT1-PE antibody (BD Biosciences) or STAT1-Alexa Fluor 647 antibody (BD Biosciences) in 10% FCS/PBS for 1 h at 4 °C. After washing three times cells were analyzed on a FACS Canto cytometer (BD Biosciences). Histogram overlays were performed using the Weasel.jar software. FACS analysis with propidium iodide was summarized in [10].

2.6. Antibodies, drugs and chemicals

This information can be found in the Supplementary materials.

2.7. Antiviral assay

A detailed description for this method can be found in the Supplementary materials.

2.8. Preparation of cell lysates, immunoprecipitation, immunoblotting, ABCD-assay (Avidin–Biotin-Coupled DNA-Assay) and EMSA (electrophoreticmobility shift assay)

These methods were described recently [5,10]. ABCD and EMSA assays were performed with the GAS site containing oligonucleotides 5-GAGAC TCAGTTTCCCGTAAATCGTCCAGTTTCCCGTAAAGACTATGC-3 and 5-GCATAGTCTTTA CGGGAAACTGGACGATTTACGGGAAACTGAG-TCTC-3 or irrelevant oligonucleotides.

3. Results

3.1. Acetylation impairs IFNγ-induced phosphorylation of STAT1

HDACi and the knock-down of individual HDACs can induce STAT1 acetylation blocking STAT1 phosphorylation [5,13,17,19]. These data were collected with transformed human cells and murine tissues. We exposed various cell lines and primary cells to agents causing cellular protein hyperacetylation. Pretreatment with the pan-HDACi trichostatin A (TSA) led to diminished STAT1 phosphorylation in freshly

isolated human umbilical vein endothelial cells (HUVECs) exposed to IFNs (Fig. 1A). This experiment also revealed that while IFNγ only induced STAT1 phosphorylation, IFNα evoked phosphorylation of STAT1, -2, and -3. Of note, TSA specifically blocked the phosphorylation of STAT1 and not of other STATs induced by IFNα (Fig. 1A). This result demonstrates that there is no general defect in IFN-dependent JAK-STAT pathway in the presence of HDACi. Equal efficacy of TSA in cells treated with IFNα or IFNγ was verified with immunoblots detecting acetylated histones.

Pretreatment of human embryonic kidney cells (293T) with TSA, likewise prevented STAT1 phosphorylation in response to IFNγ or IFNα (Fig. 1B). We could also observe this effect in IFNγ stimulated HCT116 colon cancer cells and in MCF7 breast cancer cells (Supplemental Fig. S2). Caspase-3 immunoblots of HUVEC and 293T cell extracts (Fig. 1A and B) and FACS analyses of Caspase-3 null MCF7 cells (Supplemental Fig. S3) rule out the cytotoxicity of TSA or IFNs as a reason for poor p-STAT1 signals in HDACi-treated cells.

Hematopoietic cells are the major cell relevant for immune responses and inflammation. Intracellular FACS staining with primary bone marrow cells (BMC), bone marrow-derived dendritic cells (BMDC), and NB4 promyelocytic leukemia cells showed that HDACi inhibit IFNγ-induced phosphorylation of STAT1. Pretreatment with the clinically relevant HDACi valproic acid (VPA) and MS-275 impaired IFNγ-induced p-STAT1 in these cells, but did not reduce STAT1 protein levels (Fig. 1C).

The results we show in Fig. 1 are consistent with other studies concluding that HDACi induce protein acetylation and counteract STAT1 phosphorylation [5,8,13–17,19].

3.2. STAT1 acetylation attracts the phosphatase TCP45

In order to detect acetylation of STAT1 directly in 293T cells exposed to IFNγ, we immunoprecipitated STAT1 under stringent conditions, and added TSA to preserve acetylation. Western blotting with pan-acetyl-lysine antibodies detected acetylated STAT1 (Fig. 2A). Since CREB-binding protein (CBP) is the histone acetyltransferase for STAT1 [5,8,10], we tested its functionality in 293T cells by assessing its autoacetylation [28]. Indeed, in these cells CBP is competent for acetylation of itself (Fig. 2B) and of STAT1 (Fig. 2A).

Acetylation of STAT1 after treatment with IFNγ (Fig. 2A) should prevent its tyrosine phosphorylation upon re-stimulation. Therefore, we tested whether HDACi-induced latency of STAT1 phosphorylation could be confirmed in IFNγ prestimulated 293T cells. Repeated stimulation with this cytokine did indeed not permit phosphorylation of

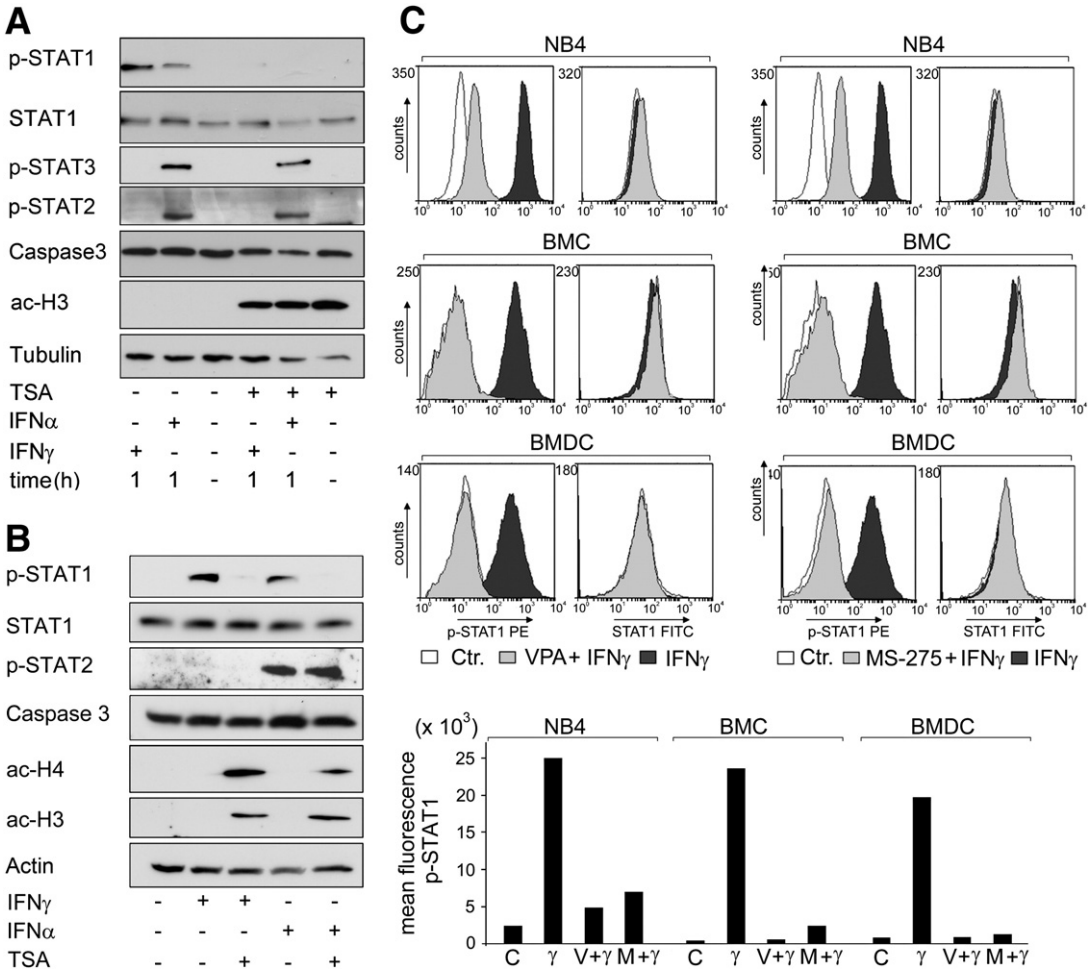

Fig. 1. IFNγ induces phosphorylation and acetylation of STAT1. (A) HUVECs were stimulated with IFNα/γ (+; 1 h) and pretreated with TSA (+; 16 h). STAT1 and p-STAT levels were determined by immunoblot. Tubulin served as loading control, acetyl-histone H3 verified TSA activity and intact caspase 3 ensured vital cells. (B) 293T cells were treated with IFNγ or IFNα for 30 min (+). Cells were pretreated with TSA (+; 1 μM; 16 h). P-STAT1/p-STAT2 levels were analyzed by Western blot. Acetyl-histone H4 proved TSA efficacy; full-length Caspase-3 marks intact cells. (C) Intracellular FACS staining of p-STAT1 revealed inhibitory effect of HDACi on p-STAT1 level in NB4 cells, BMC and BMDC. Quantification of mean fluorescence is shown in the lower panel; please note the logarithmic scale for FACS profile overlays. Cells were treated with VPA (V) or MS-275 (M) for 24 h and afterward stimulated with IFNγ (γ; 30 min); (C) untreated.

acetylated STAT1 (Fig. 2C). Previous reports also found that dephosphorylation of STAT1 by TCP45 determines the extent of IFN signaling, independent of the kinase and receptor status [5,24].

IFNα and IFNγ cause phosphorylation and nuclear translocation of STAT1. We asked if these processes were necessary for its acetylation. We compared STAT1^{Y701F} (which lacks the STAT1 tyrosine phosphorylation site Y701), import deficient STAT1^{L407A,L409A} (which can be tyrosine phosphorylated at Y701 [29]), and wild-type STAT1. The fact that the STAT1 mutant molecules did not become acetylated in response to IFN argues that both, tyrosine phosphorylation and nuclear translocation of STAT1 are necessary for its acetylation (Supplemental Fig. S4).

After prolonged IFN stimulation, acetylated STAT1 accumulates in the cytosol in a complex with TCP45 and CBP [5,8]. These data were collected with a substrate trapping form of TCP45. To refine these analyses and to test whether class I and II IFNs induce STAT1-TCP45 complexes, we performed fluorescence energy transfer (FRET) analyses in 293T cells. These experiments showed that both types of IFNs strongly enhance the STAT1/TCP45 interaction in the cytosol (Fig. 2D). We sum up that IFNγ-induced STAT1 acetylation after its phosphorylation and subsequent association with TCP45 appear causally linked to STAT1 latency.

3.3. Mutations mimicking acetylation lead to inactivation of STAT1

STAT1^{QQ} has lysine (K) to glutamine (Q) exchanges at K410 and K413, which are located within the STAT1 DNA binding domain (DBD) (Fig. 3A). Glutamine resembles acetylated lysine and, when introduced in the DBD, phenotypically copies/mimics acetylated STAT1 [5]. Thus, STAT1^{QQ} serves as a model for acetyl-STAT1. When we applied IFNγ to U3A cells transfected with STAT1 or STAT1^{QQ}, we could not detect tyrosine phosphorylation of STAT1^{QQ}. We made similar observations for STAT1 molecules imitating single acetylation at K410 or K413 (STAT1^{410Q}/STAT1^{413Q}). As a control, we exchanged K410 and K413 with arginine (R), which cannot become acetylated. As anticipated, STAT1^{RR} was phosphorylated as wild-type STAT1 (Fig. 3B).

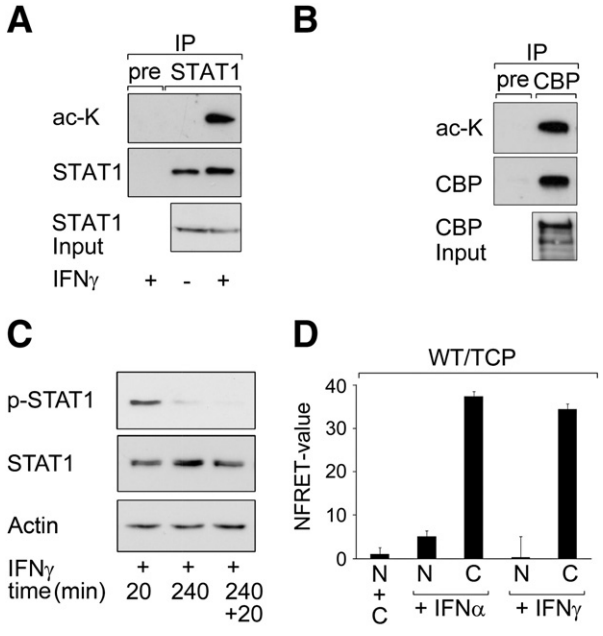

Fig. 2. Acetylation of STAT1 and interaction with TCP45. (A) STAT1 was precipitated from 293T cells treated with IFNγ (+; 4 h) and analyzed by immunoblot (IP, immune precipitation; pre, pre-immune serum IP; ac-K, anti-acetyl-lysine). (B) IP of CBP from 293T cells. Acetylation of CBP was tested using a pan-specific anti-ac-K antibody for Western blot analyses. (C) 293T cells were incubated with IFNγ (+; 4 h) and were re-stimulated for 20 min with IFNγ. Levels of p-/total STAT1 were determined by immunoblot. (D) Analysis of FRET microscopy showing interactions between TCP45 and STAT1 in 293T cells. Error bars are SEM for 25–27 cells per condition; nucleus (N), cytosol (C).

FRET analyses revealed that, compared to wild-type STAT1, STAT1^{QQ} was more closely associated with TCP45 (Fig. 3C). These observations suggest that poor tyrosine phosphorylation of STAT1^{QQ} stems from rapid dephosphorylation of the STAT1 mutant.

Consistent with the fact that the IFN-induced nuclear import of STAT1 requires its tyrosine phosphorylation, STAT1^{QQ} did not accumulate in the nucleus in response to IFNγ (Fig. 3D). The export inhibitor LMB prevented cytosolic re-appearance of IFNγ-induced STAT1, and it failed to retain STAT1^{QQ} in the nucleus (Supplemental Fig. S5). This result confirms that poor tyrosine phosphorylation of STAT1^{QQ} prevents its translocation to the nucleus upon IFN treatment. Moreover, STAT1^{QQ} could not activate a luciferase reporter containing an IFN-responsive GAS-site, but acetylation-deficient STAT1^{RR} induced this reporter more efficiently than the wild-type STAT1 did (Fig. 3E). Expression analysis of the endogenous IFN-inducible STAT1 target gene *UBCH8*, which contains GAS- and ISRE-sites [5], demonstrated that STAT1^{QQ} also lacked transcriptional activation potential on an endogenous STAT1 target gene (Fig. 3F).

These results demonstrate that STAT1^{QQ} interacts with TCP45, is rapidly dephosphorylated, and is unable to enter the nucleus to induce gene expression.

3.4. PTP inhibition cannot restore IFNγ signaling via STAT1 lacking a functional DBD

Since acetylation marks STAT1 for TCP45-binding, we asked whether PTP inhibition and altering of the cellular balance of phosphorylating versus dephosphorylating enzymes could activate STAT1^{QQ} in reconstituted U3A cells. The broad range PTP antagonist vanadate permitted phosphorylation of this STAT1 mutant in cells exposed to IFNγ (Fig. 4A). Similarly, overexpression of the IFNγ-receptor-associated kinase JAK2 and shRNA-mediated attenuation of TCP45 allowed phosphorylation of STAT1^{QQ} (Fig. 4B). These findings corroborate previous data demonstrating that STAT1^{QQ} has a cellular localization and structure comparable to wild-type STAT1. Hence, STAT1^{QQ} principally interacts with receptors and JAKs [5]. Surprisingly, STAT1^{QQ} became phosphorylated upon addition of IFNγ plus vanadate, but could not be recovered with a GAS oligonucleotide (Fig. 4C). This finding was unexpected as we previously found that STAT1^{QQ} can bind cognate DNA in U3A cells treated with IFNα and vanadate [5]. Therefore, we considered that IFNα and IFNγ cause different effects that cannot be solely explained by STAT1 phosphorylation.

Unlike IFNα, IFNγ only induces phosphorylation of STAT1 homodimers in many cellular systems [9]. Since IFNγ can cause phosphorylation of STAT3 in certain cells, we analyzed if our IFNγ-treated U3A cells only contain p-STAT1 homodimers or also p-STAT3 which is able to form heterodimers with STAT1. We noted that p-STAT3 was not detectable in U3A cells exposed to IFNγ plus vanadate (Supplemental Fig. S6). Therefore, we conclude that irrespective of their IFNγ/vanadate induced tyrosine phosphorylation acetylation-mimicking STAT1^{QQ} homodimers cannot bind target DNA.

As anticipated from its poor DNA binding, p-STAT1^{QQ} did not activate a GAS-Luc reporter in reconstituted U3A cells incubated with IFNγ plus vanadate (Fig. 4D). As p-STAT1^{QQ} could still enter the nucleus (Supplemental Fig. S7), defective nuclear functions – i.e. specifically a lack of DNA binding – seem to prevent productive IFN signaling via p-STAT1^{QQ}.

3.5. The number of acetylation-mimicking mutations in STAT1 affects signaling

Similarly to STAT1^{QQ}, STAT1^{410Q} and STAT1^{413Q} remain largely unphosphorylated in the presence of IFNγ (Fig. 3B). This situation allowed us to analyze whether individual lysine to glutamine mutations in the acetylation module impair the STAT1 DBD. We applied IFNγ to U3A cells expressing STAT1^{410Q} or STAT1^{413Q} and noticed

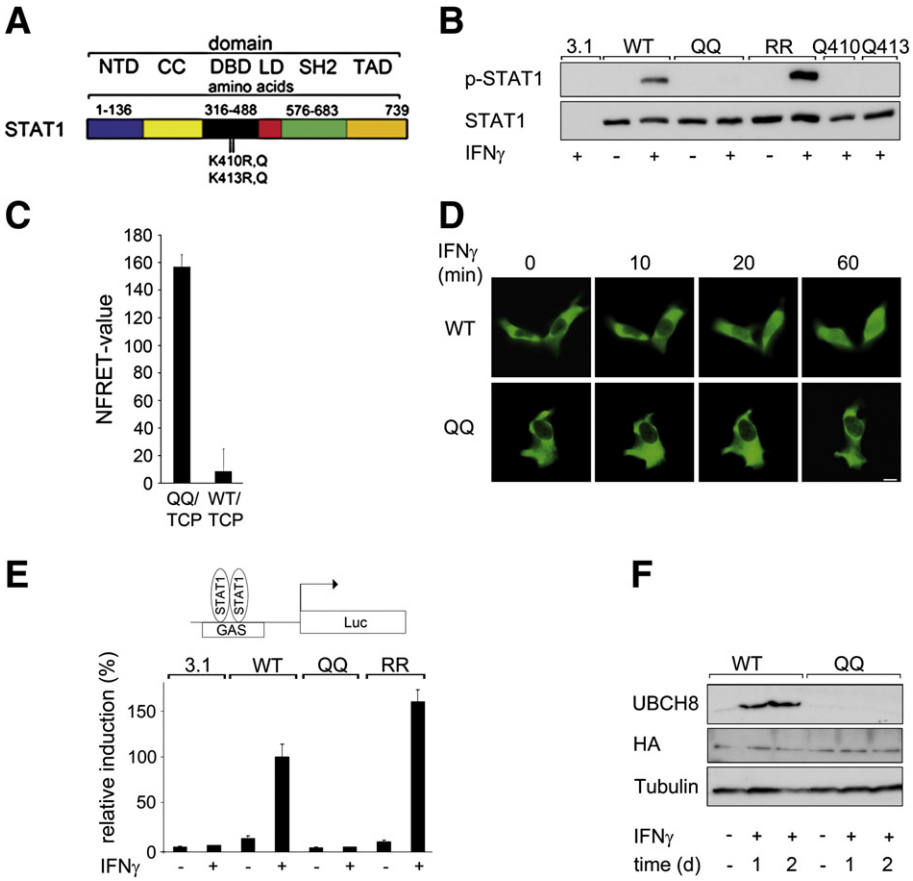

Fig. 3. STAT1 acetylation affects signaling. (A) Schematic view of STAT1: N-terminal (NTD), coiled coil (CC), DNA binding (DBD), linker (LD), Src homology 2 (SH2) and transactivation (TAD) domains. Mutated sites are accentuated. (B) U3A cells transfected with vectors for STAT1 (WT, wild-type), STAT1^{K410,413Q} (QQ), STAT1^{K410,413R} (RR), STAT1^{K410Q} (Q410), HA-STAT1^{K413Q} (Q413) or pcDNA3.1 (3.1) were treated with IFNγ (+) for 30 min. P-STAT1/STAT1 levels were determined by immunoblot. In few experiments a faint phosphorylation at Y701 was detectable after very long blot exposures >1 h. (C) FRET analysis detected interactions between STAT1 (QQ) and TCP45; 25–27 cells were analyzed; error bars: SEM. (D) Translocation of EGFP-STAT1 (WT/QQ) was assessed by live cell time-lapse fluorescence microscopy. U3A cells were treated with IFNγ (0–60 min); scale bar 10 μm. (E) GAS-luciferase assay quantified transcriptional activity of STAT1 versions in U3A cells. IFNγ-induced reporter activation (24 h treatment) by wild-type STAT1 is set as 100%; $p<0.001$ over controls. (F) U3A cells stably expressing HA-STAT1 (WT/QQ) after selection with Neomycin (500 μg/ml) were incubated with IFNγ (+; 1–2 d). Protein levels were determined by immunoblot.

their tyrosine phosphorylation in the presence of IFNγ and vanadate (Fig. 5A). In contrast to p-STAT1^{QQ} (Fig. 4C), individually mutated p-STAT1^{410Q} and p-STAT1^{413Q} bound to GAS DNA (Fig. 5A). Accordingly, transcriptional activation of the GAS reporter could be induced by these STAT1 variants (Fig. 5B). This finding suggests that the number of site-specific acetylation-like sites in the STAT1 DBD controls whether IFNγ-induced STAT1 homodimers activate signaling.

3.6. Heterodimerization can compensate for loss of activity by STAT1 acetylation

Since IFNα can cause phosphorylation of all seven STATs, a functional rescue of p-STAT1^{QQ} by heterodimerization with other p-STATs appears plausible. The fact that STAT3 has often been detected with STAT1 on GAS sites [30–33], points to a crosstalk of these STATs on DNA. To test this, we first analyzed IFNα-induced phosphorylation of endogenous STAT1/STAT3 and checked for their presence on the GAS consensus oligonucleotide. Precipitation of STAT3 confirmed specific interactions of p-STAT1 with p-STAT3 in 293T cells treated with IFNα (Fig. 6A and B). In addition, IFNα triggered binding of both molecules to GAS oligonucleotides (Fig. 6C). These data are congruent with previous results collected with EMSAs and chromatin immunoprecipitations [30–33].

In order to test the hypothesis of a rescue of p-STAT1^{QQ} *in trans*, we compared its reactions to the two types of IFNs side-by-side. Only when U3A cells had been pretreated with vanadate, IFNα though not IFNγ, led to binding of STAT1^{QQ} with amply phosphorylated STAT3 on a GAS oligonucleotide (Fig. 6D). The failure of IFNγ to induce p-STAT3 in U3A cells (Supplemental Fig. S6) correlated with a lack of DNA binding of p-STAT1^{QQ}. These results suggest that heterodimer formation with p-STAT3 upon incubation with IFNα allows STAT1^{QQ} to recognize target DNA.

STAT3 appears with STAT1 in heterodimeric complexes, but we cannot exclude contributions from other STATs. To further test the putative role for STAT3 in rescuing STAT1^{QQ} functions, we checked whether interleukin-6 (IL6), a cytokine that induces STAT3 phosphorylation [30–33] can influence the DNA binding ability of STAT1^{QQ}. Remarkably, EMSAs revealed that IL6 plus vanadate allowed the recruitment of STAT1^{QQ} to a GAS oligonucleotide (Fig. 6E). Whereas activation of STAT3 was verified by STAT3-specific upshift in band shift assay, cross-phosphorylation of STAT1 in U3A cells by IL6/vanadate could be ruled out as an alternative explanation (Supplemental Fig. S8). Moreover, IFNγ/vanadate specifically stimulated STAT1^{QQ} (Fig. 6E and Supplemental Fig. S6) but also failed to promote DNA binding of p-STAT1^{QQ} in EMSAs (Fig. 6D and E). These findings suggest that IL6-induced p-STAT3 can be a p-STAT1^{QQ} heterodimer partner to mediate contact with DNA.

3.7. Mimicking STAT1 acetylation in the context of anti-viral defense

These data prompted us to examine whether STAT1^{QQ} generated any protective effects against vesicular stomatitis virus (VSV)

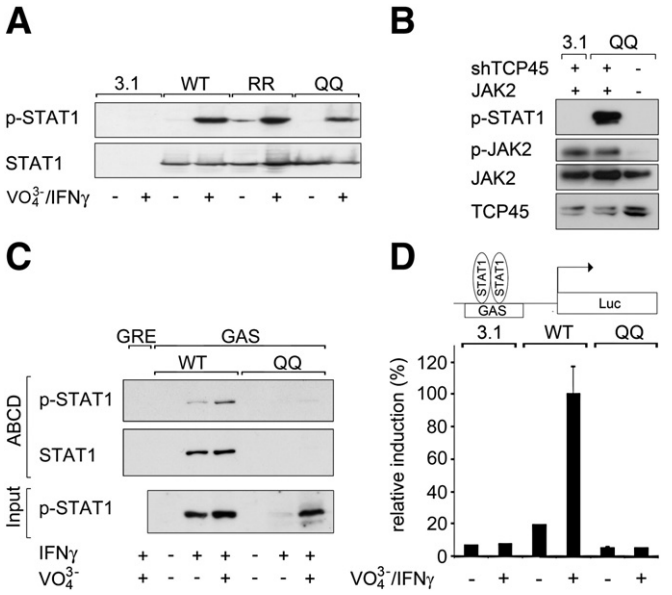

Fig. 4. Phosphorylation and DNA binding of acetylation mimicking STAT1. (A) U3A cells, transiently expressing STAT1 (WT, wild-type), STAT1^{K410,413R} (RR), STAT1^{K410,413Q} (QQ) or pcDNA3.1 (3.1), were pretreated with vanadate (30 min) followed by IFNγ stimulation (30 min, +). P-STAT1 and STAT1 were analyzed by immunoblot. (B) U3A cells were transfected with JAK2 cDNA (+), shRNA for TCP45 (+, shTCP45) and STAT1^{K410,413Q} (QQ) or empty vector (pcDNA3.1). Protein levels were analyzed by immunoblot. (C) ABCD assay with U3A cells stably expressing STAT1 (WT/QQ) were used to investigate DNA binding capacity at GAS element containing oligonucleotides (GAS). Cells were stimulated with vanadate (+, 30 min) and IFNγ (+, additional 30 min). Glucocorticoid response element (GRE) was used as negative control. (D) GAS-Luc assay showed transcriptional activity of STAT1 (WT/QQ) in U3A cells, treated with vanadate and IFNγ for 6 h (+). Statistical significance over controls is $p<0.001$.

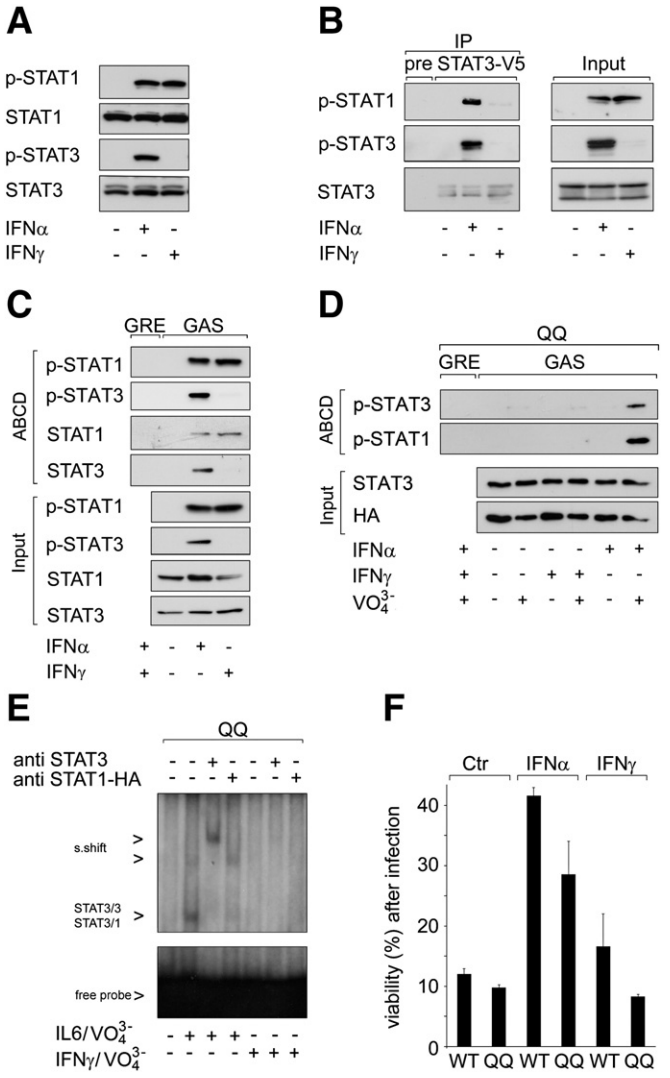

Fig. 6. IFNα/IFNγ provoke activation of STAT proteins differentially. (A) 293T cells were exposed to IFNα/γ for 30 min (+). Lysates were analyzed by immunoblot. (B) 293T cells transfected with STAT3-V5 were stimulated with IFNα/γ for 30 min (+). Immunoprecipitation (IP) against V5 was probed for p-STAT1 (pre, control IgG IP). (C) DNA binding of STAT1/STAT3 was assessed by ABCD assay in 293T cells. Cells were treated as in (B). (D) U3A cells transfected with HA-STAT1^{K410,413Q} (QQ) were pretreated with vanadate (30 min) and stimulated for 1 h (IFNα/γ). DNA binding of p-STAT1/p-STAT3 was investigated by ABCD-assay. (E) DNA binding of HA-STAT1^{K410,413Q} (QQ) was investigated by EMSA. U3A cells were transfected with STAT1 and wild-type STAT3 constructs followed by stimulation with IL6 or IFNγ (+) and vanadate. Protein-oligonucleotide complexes were shifted by using antibodies for STAT3 or HA-tag (+). (F) Antiviral protection mediated by STAT1 (WT) and STAT1^{K410,413Q} (QQ) was determined by cytopathic effect assay (Ctr, untreated control cells). Stably transfected U3A were stimulated with IFNα/γ for 24 h before infection with VSV (MOI 1.0).

infection. VSV is highly sensitive to IFN and routinely used to assay IFN-dependent antiviral activities [8,20]. IFNα significantly protected U3A cells stably reconstituted with wild-type STAT1. IFNγ protected such cells to a lesser extent, which agrees with the finding that most anti-viral genes are induced by IFNα-induced trimeric STAT1-STAT2-IRF9/ISRE complexes. Of note, STAT1^{QQ} provided a notable defense against this virus for U3A cells, stimulated with IFNα but not upon treatment with IFNγ (Fig. 6F). These findings correspond to our in vitro DNA binding and transcriptional activation assays, which show that IFNα partially rescues inactive STAT1^{QQ}. Apparently, STAT1 with a functionally impaired DBD (STAT1^{QQ}) leading to rapid dephosphorylation, can still induce anti-viral programs when cells are exposed to IFNα and VSV. These findings reveal that cellular defense against a complex stimulus as VSV is, not only depends on an active STAT1 DBD.

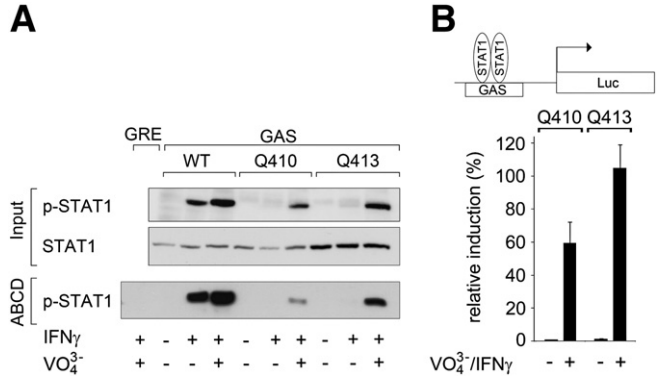

Fig. 5. Site specific mutations of acetylation-mimicking STAT1. (A) DNA binding capability of STAT1^{K410Q} (410Q) and STAT1^{K413Q} (413Q) from lysates of transfected U3A cells was tested with ABCD assay. (B) 410Q and 413Q activate the GAS-Luc reporter. Transiently transfected U3A cells were stimulated with vanadate and IFNγ (+, 24 h). Values for Q413 were set as 100%.

We conclude that via differential induction of STAT1 homo- or heterodimer phosphorylation, IFNα and IFNγ can discriminatively affect DNA binding of STAT1 lacking a functional DBD (Fig. 7; see Discussion for further details).

4. Discussion

IFNs signal through STAT1, a pivotal regulator of cell fate, growth, and immunity. STAT1 is regulated by phosphorylation,

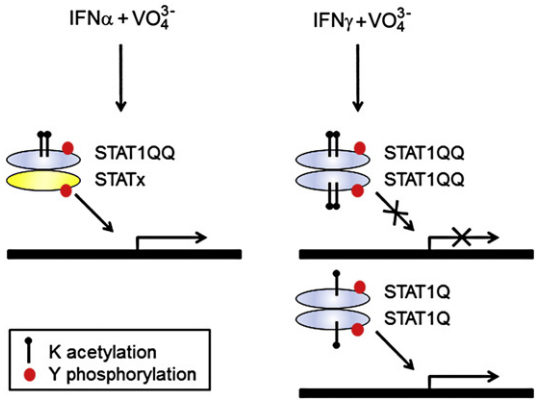

Fig. 7. IFNα/IFNγ provoke activation of STAT proteins differentially. Multiple levels control STAT1 via acetylation including phosphorylation–acetylation switch cassettes and homo/heterodimer formation. IFNα induced STAT1QQ heterodimers are functional. However, IFNγ evoked phosphorylated $STAT1^{QQ}$ homodimers are incompetent for DNA binding. Vanadate is necessary to inactivate TCP45 that rapidly binds and dephosphorylates acetylation-mimicking STAT1. HDACi treatment or K → Q mutation changes the functional outcome of STAT1. IFNγ evoked STAT1 homodimers are incapable for GAS (IFNγ-activated site) binding. In case of IFNα, other STATs (STATx) can compensate the inactive STAT1. STAT2 enables ISRE (interferon stimulated response element) and STAT3 GAS binding of the heterodimer. STAT1 carrying one acetylation cassette at K410 or K413 are dephosphorylated by TCP45, but still capable to induce IFN-dependent STAT1 signaling upon inhibition of this effector molecule. Thus, STAT1 post-translational modification cassettes functionally link upstream and downstream signaling events and interaction partners.

acetylation, and additional posttranslational modifications [25]. Chemically induced STAT1 acetylation and K → Q mutations mimicking acetylation within the STAT1 DBD at K410/K413 suggest that acetylation impairs DNA binding and facilitates dephosphorylation of STAT1.

The lysine residues K410/K413 are in the surface-exposed DBD and in contact with DNA. Their acetylation may relax p-STAT1/DNA contacts to facilitate structural rearrangement presenting pY701 to TCP45 [5,12]. This agrees with the notion that acetylated STAT1 and STAT1 with mimicked acetylation within its DBD can hardly be phosphorylated [5] and that DNA binding controls STAT1 inactivation [29]. Albeit K410 and K413 are not the only residues mediating DNA contact [12], simulating constitutive acetylation at both of these sites precludes high affinity binding of p-STAT1 to DNA. This finding is congruent with the rapid dephosphorylation of acetylation-mimicking STAT1 and its poor ability to bind cognate DNA.

We now reveal that the outcome of STAT1 acetylation depends on whether STAT1 is in homo- or heterodimer complexes. Co-induction of STAT phosphorylated heterodimers, e.g. by IFNα or IL6 can position DBD mutant STAT1 on DNA. These data are in agreement with our previous work [5]. In these analyses we used IFNα to induce STAT1. Accordingly, we obtained results for STAT1 homo- and heterodimers, i.e. for STAT1 complexes that can bind DNA in a heterodimer complex and STAT1 homodimers that remain inactive even when they are tyrosine phosphorylated.

We now decipher more precisely how STAT1 homodimers behave upon HDACi-induced acetylation and when acetylation is mimicked in the STAT1 DBD. Our data extend the finding that type I IFNs induce the STAT1–STAT2–IRF9 complex that can overcome an inability of STAT1 to bind chromatin due to an import defect (L407A mutation) [7]. We extend these findings by analyses within the context of STAT1 acetylation, and suggest that a "piggy-back" mechanism can operate for STAT1. Interestingly, similar observations were made for dimerization-dependent mechanisms regulating nuclear hormone receptor signaling. In the case of nuclear receptor response elements only one of two half sites needs to be covered perfectly, i.e. just one of the heterodimer partners must recognize it optimally. This mechanism allows gradually tuning and varying the cellular ligand response [34]. Notably, we reveal that cellular defense against the complex stimulus VSV not only depends on the DNA binding capacity of STAT1. Perhaps the observed inhibition of innate cellular immunity by HDACi also depends on inactivating effects beyond STAT1 acetylation [18,20,35]. Therefore, our novel data integrate into current research gaining new insights into the biology of STATs.

The model shown in Fig. 7 summarizes and integrates our findings. It particularly accentuates the differences between class I/II IFNs and of STAT1 with acetylation–phosphorylation cassettes, i.e. acetylated lysine residues that prevent phosphorylation of a critical tyrosine residue. It is equally noteworthy that STAT1 and STAT2 as well as STAT3 have frequently been found together on promoters of IFN-responsive genes [2,3,7,30–33]. Moreover, STAT3 can associate with STAT1 on chromatin, and these STATs can substitute each other in matching null cells [30–33]. Our new findings point out that STAT acetylation should not be viewed as an isolated parameter, but instead in a context-dependent fashion regarding other STAT family members. Curiously, STAT2 and STAT3 do not contain lysine residues at sites corresponding to STAT1 K410/K413 [25], and are not inactivated upon hyperacetylation [8,36,37]. Thus, they may well provide functions to acetylated STAT1 *in trans*. Acetylation of STATs could also establish positive and negative interactions between STAT proteins. Furthermore, we disclose that the number of site-specific acetylation/tyrosine phosphorylation cassettes in STAT1 is important. Similar data were collected for the tumor suppressor p53, in which acetylation of different lysines controls acetylation/serine phosphorylation cassettes [38].

While mutation of K410/K413, located within the putative STAT1 NLS/NES, impairs IFN-induced nuclear translocation of STAT1, forced phosphorylation with vanadate permits nuclear transit of $STAT1^{QQ}$ ([5] and this study). Apparently, other sites can dictate translocation of STAT1 via importins. L407 has repeatedly been found to be critical for nuclear entry of p-STAT1 [7,39]. Moreover, only STAT1 with mutated L407/L409, and not STAT1 with alterations at K410/K413, displays strictly cytosolic localization [29]. Data from STA-1 (STAT orthologue) of the nematode *Caenorhabditis elegans* equally show that the DBD is not always involved in the activation and nuclear accumulation of STAT molecules [40]. Truly, the STAT1 DBD is an essential feature of JAK-STAT signaling in vertebrates. We speculate that at the same time it may have evolved to exert a negative feed-back regulation on STAT tyrosine phosphorylation, possibly also within the context of acetylation.

Cytokines and pharmacological agents can affect STAT acetylation and signaling [4,25]. We used the class I HDAC-specific HDACi VPA and MS-275 (VPA blocks HDAC1/-2/-3/-8; MS-275 targets HDAC1/-2/-3) [41]. Our results demonstrate that such HDACs, which are often overexpressed in tumors [25], are key players in the antagonistic relationship between STAT1 phosphorylation and acetylation. The class II HDAC family member HDAC4 can equally antagonize STAT1 acetylation [17]. The observation that several HDACs control STAT1 acetylation argues for the relevance of this posttranslational modification [25]. On the other hand, only one acetyltransferase, CBP, has been found to catalyze STAT1 acetylation [5,8,10].

The cell cycle regulator $p16^{INK4a}$ is an additional control protein for the STAT1 phospho-acetyl-switch and there is an increased expression of CBP in $p16^{INK4a}$ negative cells [16]. Since STAT1 acetylation can reduce cellular vitality [5,10,25], a loss of $p16^{INK4a}$ might be compensated by increased STAT1 acetylation facilitating apoptosis. More work has to be done to clarify whether such a fail-safe mechanism against oncogenesis exists. Interestingly, the acetylation-dependent inhibition of STAT1 phosphorylation equally determines the sensitivity of ovarian cancers toward the chemotherapeutic agent cisplatin [17]. Moreover, conditions of sepsis, inflammation

and graft-versus-host disease are also ruled by STAT1 phosphorylation and acetylation [13,14]. The functional relevance of STAT1 acetylation is broader than initially anticipated and the STAT1 phosphorylation–acetylation switch has several regulators warranting further investigation.

5. Conclusions

IFNα activates several STAT proteins, whereas IFNγ induces phosphorylation of STAT1 homodimers.

Whereas acetylation negatively regulates STAT1 activation by IFNγ and IFNα, STAT2 and STAT3 can be phosphorylated in the presence of HDACi.

Intact phosphorylated heterodimer partners can compensate for the inactivation of STAT1 by acetylation.

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Supplemental section

(**S1**) A part of the DNA sequences of STAT1 wild-type (WT) and STAT1^{K410,413Q} (QQ) is shown. Lysine 636 is maintained in both constructs.

(**S2**) HCT116 colon cancer-derived cells and Caspase 3$^{-/-}$ MCF7 breast cells were preexposed to 1 μM TSA for 16 h and afterwards stimulated with IFNγ for 1 h. The amount of phosphorylated STAT1, total STAT1, Tubulin and acetyl-histone H3 were determined by immunoblot analysis.

(**S3**) MCF-7 cells were analyzed for apoptosis using propidium iodide staining (PI). Cells were treated with TSA (16 h, 1 μM).

(**S4**) Prerequisites for acetylation. U3A cells were transiently transfected with STAT1 (WT; wild-type), STAT1^{Y701F} (YF) or STAT1^{L407,409A} (LLAA). STAT1 IP of U3A cell lysates was probed for acetyl-lysine by immunoblot. Cells were stimulated with IFNα for 4 h.

(**S5**) U3A cells were transfected with plasmids for EGFP-STAT1 (WT) and EGFP-STAT1 (QQ). Cells were pretreated with nuclear export inhibitor LMB, followed by stimulation with IFNγ for the times indicated. Translocation was observed by live cell time-lapse fluorescence microscopy.

(**S6**) U3A cells were pretreated for 30 min with vanadate (+) and afterwards were stimulated with IFNα/γ (+; 30 min). Protein levels of p-STAT3 and STAT3 were analyzed by immunoblot.

(**S7**) Live cell time-lapse fluorescence microscopy for EGFP-STAT1^{K410,413Q} (QQ) translocation. U3A cells were pretreated with vanadate for 60 min followed by IFNγ; scale bar: 10 μm.

(**S8**) U3A cells were transiently transfected with STAT1^{K410,K413Q} (QQ). Cells were incubated with vanadate for 30 min and additional 30 min with IFNγ or IL6 (+). STAT levels were assessed by Western blot.

Generation of primary cells

Bone marrow cells were isolated from the femur of 6 to 12 week old C57BL/6 mice (purchased from Charles River Laboratories). Mixed bone marrow cells (3X10^6 cells/ml) were cultured in RPMI 1640 medium (Biochrom AG), 10% FCS (PromoCell)/ 1% Pen/Strep (PAA Laboratories). Bone marrow-derived dendritic cells (BMDCs) cells were prepared as described by Bode et al. [1].

Antibodies, drugs and chemicals

Antibodies were purchased from Santa Cruz Biotechnology (Caspase3, sc-7272; CBP, sc-369; STAT1, sc-346/sc-417; p-STAT1, sc-7988-R; STAT2, sc-476; STAT3, sc-482; p-STAT3, sc-8059; HA, sc-7392/sc-805; GFP, sc-9996;); Sigma (Actin, A2066; FLAG, F3165; Tubulin, T5168); Roche (TCP45, CF4-1D); Covance (HA, 11-MMS-101P); Abgent (UBCH8, AP2118b); Upstate (ac-Histone H3, 06-599; JAK2, 06-255); NEB Cell Signaling (acK, 9441; p-JAK2, 4406) and Biovision (p-STAT2, 3469-100). The ac-Histone H4 antibody was described previously and drugs and chemicals were from the sources listed in [2, 3]. IFNγ and IL6 were purchased from Immunotools.

FRET microscopy and analysis

FRET microscopy was done on a Zeiss LSM510 META confocal laser scanning microscope using the 3-Filter method [4]. GFP-STAT1 was imaged with the 488 nm line of the Ar-laser (25 mW, at 10%) with emission at 505 - 530 nm; RFP-TCP45 with the 543 nm line of the HeNe-laser (1 mW at 100%) with emission above 574 nm (using the META detector) and a raw FRET-channel was acquired with the 488 nm line of the Ar-laser (25 mW, at 10%) and emission at channel 3 (Ch3) using a LP560 nm filter.

The images were analyzed with the ImageJ package (available at: http://rsbweb.nih.gov/ij/) using the PixFRET-plugin [5] for calculating bleed-through corrected FRET images (as originally described by [6]) and expression-level normalized FRET images [7]. The mean intensities of regions of interest (such as nuclei or cytosol) were determined with ImageJ and analyzed with the MS-Excel package.

Antiviral assay

The antiviral effect of IFNα and IFNγ was determined by measuring the cytopathic effect (cpe) of vesicular stomatitis virus (VSV) on stably transfected U3A cells. Cells were incubated with IFNs for 24 h in 96-Well plates. Supernatant was removed and cells were infected with VSV at a multiplicity of infection (MOI) 1. Cells were incubated with VSV for 24 h. Plates were washed carefully with PBS to remove cellular debris of necrotic cells. Remaining cells were fixed and stained with 0.2% crystal violet, 20% ethanol, and 3.5% formaldehyde in ddH_2O for 24 h. Stained cells were washed three times with water and dried. Remaining dye was extracted using lysis buffer (48% ethanol, 2% 1 N HCl, and 0.9 g sodium citrate in ddH_2O) for 20 min. Absorbance was measured on a Dynatech MR5000 plate reader at 550/630 nm. Each point of measurement was done as quadruplicate and normalized to uninfected control cells (set as 100%). Results represent arithmetical means of three independent assays.

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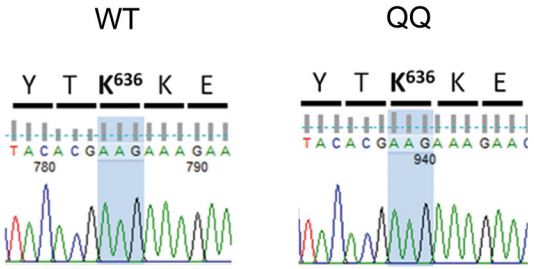
S1



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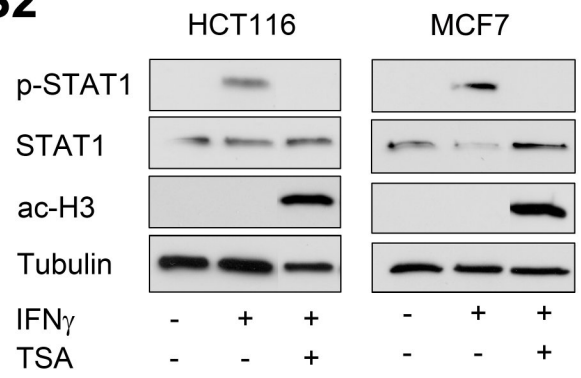

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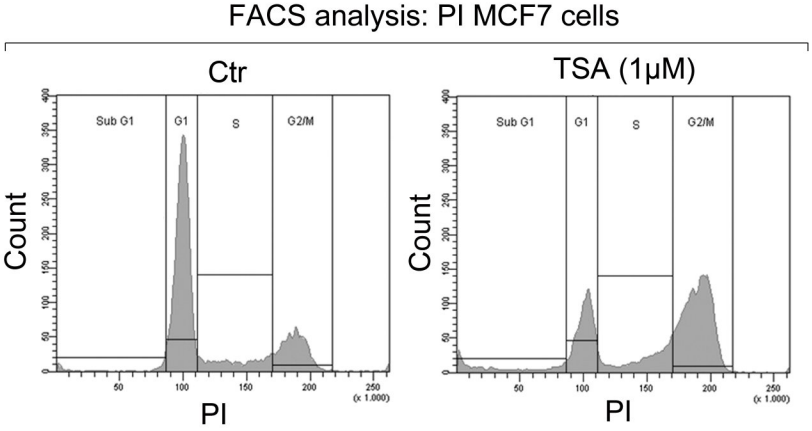

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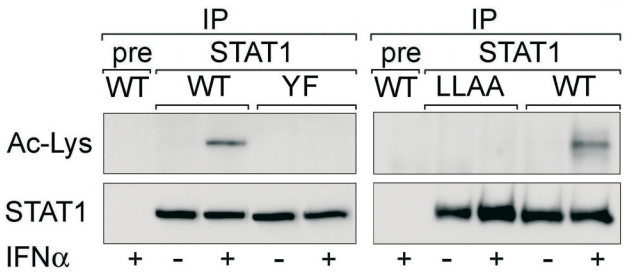

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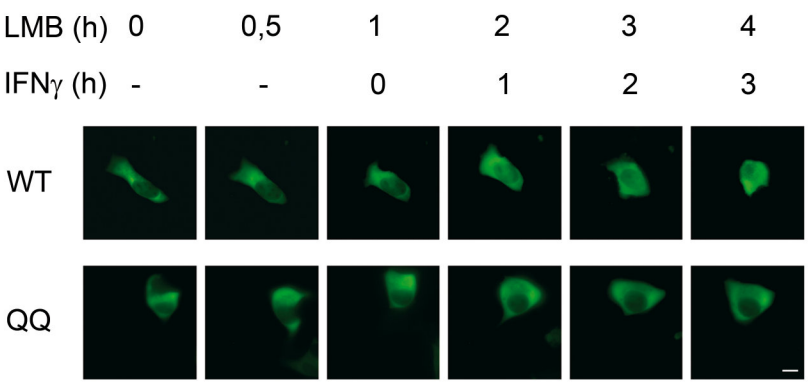

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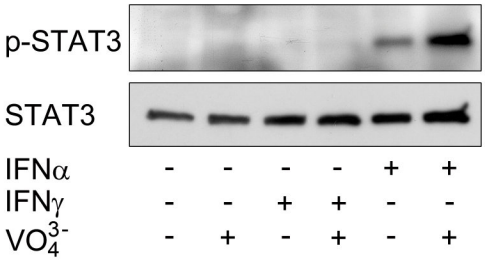

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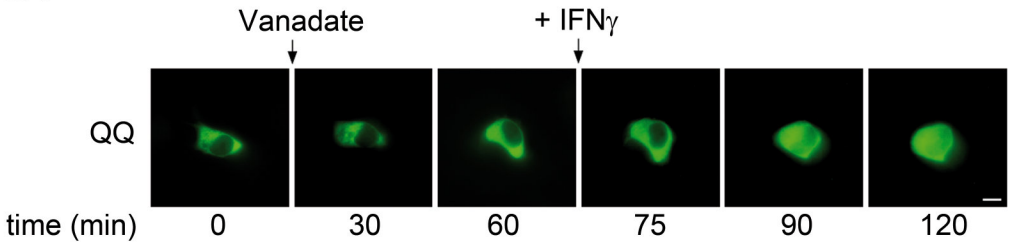

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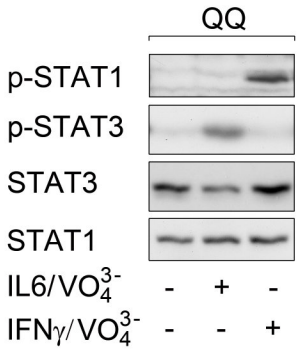

Manuskript 2: Acetylation modulates the STAT signaling code

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Survey

Acetylation modulates the STAT signaling code

Martin Wieczorek [1], Torsten Ginter [1], Peter Brand [1], Thorsten Heinzel, Oliver H. Krämer *

Center for Molecular Biomedicine, Institute for Biochemistry and Biophysics, Department of Biochemistry, Friedrich Schiller University of Jena, Hans-Knöll-Straße 2, 07745 Jena, Germany

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ABSTRACT

A fascinating question of modern biology is how a limited number of signaling pathways generate biological diversity and crosstalk phenomena *in vivo*. Well-defined posttranslational modification patterns dictate the functions and interactions of proteins. The signal transducers and activators of transcription (STATs) are physiologically important cytokine-induced transcription factors. They are targeted by a multitude of posttranslational modifications that control and modulate signaling responses and gene expression. Beyond phosphorylation of serine and tyrosine residues, lysine acetylation has recently emerged as a critical modification regulating STAT functions. Interestingly, acetylation can determine STAT signaling codes by various molecular mechanisms, including the modulation of other posttranslational modifications. Here, we provide an overview on the acetylation of STATs and how this protein modification shapes cellular cytokine responses. We summarize recent advances in understanding the impact of STAT acetylation on cell growth, apoptosis, innate immunity, inflammation, and tumorigenesis. Furthermore, we discuss how STAT acetylation can be targeted by small molecules and we consider the possibility that additional molecules controlling STAT signaling are regulated by acetylation. Our review also summarizes evolutionary aspects and we show similarities between the acetylation-dependent control of STATs and other important molecules. We propose the concept that, similar to the 'histone code', distinct posttranslational modifications and their crosstalk orchestrate the functions and interactions of STAT proteins.

Contents

1. Introduction

1.1. The STAT protein family and its activation

STAT proteins are latent cytoplasmic transcription factors that can be induced by cytokines and growth factors [1,2]. There are seven STATs (STAT1,-2,-3,-4,-5a,-5b,-6) in mammalian cells and all of them have individual and overlapping functions and regulators

* Corresponding author. Tel.: +49 3641 949362; fax: +49 3641 949352.
E-mail address: Oliver.Kraemer@uni-jena.de (O.H. Krämer).
[1] These authors contributed equally.

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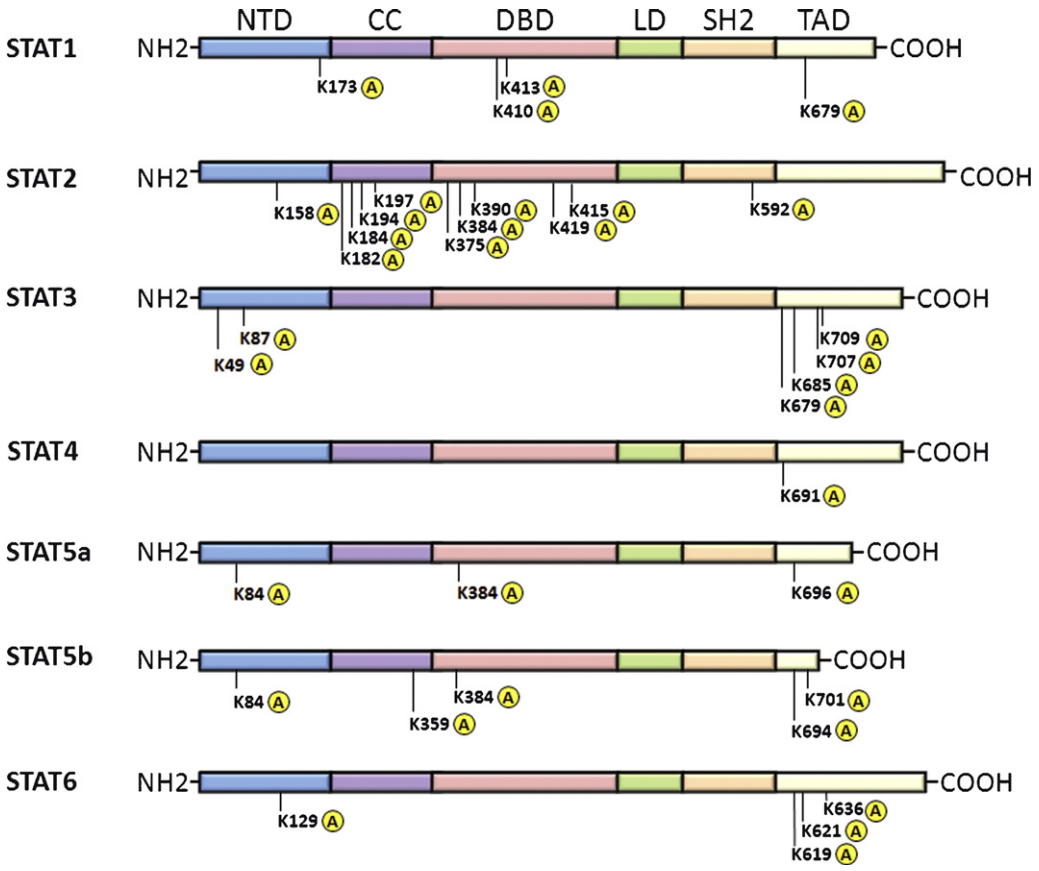

Fig. 1. Posttranslational modifications define the STAT signaling code. Data available suggest that STATs are controlled by various posttranslational modifications. This situation is reminiscent of the "histone code hypothesis" which says that different posttranslational modifications of histones influence each other; A, acetylation; K, lysine residue. Shown are acetylation sites found for the different STAT family members.

in vivo [3]. STAT proteins consist of an N-terminal domain (NTD), a coiled-coil domain (CC), a β-barrel DNA binding domain (DBD), a linker domain (LD), an SH2 domain, and a C-terminal transactivation domain (TAD) [4] (Fig. 1).

In the canonical STAT activation pathway cytokines and growth factors trigger the association of receptors to activate Janus tyrosine kinases (JAKs, JAK1,-2,-3 and TYK2) [5,6]. Binding of STATs to the tyrosine phosphorylated receptors then allows the phosphorylation of STATs by JAKs. Two phosphorylated STATs associate with each other by phospho-tyrosine/SH2 domain interactions [1,3]. Such dimers enter the nucleus and induce expression of their target genes. Tetramers and octamers of STATs were also found to control transcription [7,8]. Subsequent parallel to anti-parallel transitions allow presentation of the phosphorylated tyrosine residue for signal termination by phosphatase-dependent dephosphorylation [1,9]. These processes require conformational changes involving protein interactions within the DBD, CC, NTD, and SH2 domain [1]. Prior to activation STATs already associate with other STATs *via* their N-termini and such unphosphorylated STATs already regulate a subset of STAT-dependent genes [3].

The precise control of JAK-STAT signaling is a prerequisite for the adaptation to changes in the environment and for the maintenance of homeostasis [6]. Numerous diseases are linked to disturbed JAK-STAT signaling nodes causing failed or unbalanced immunological functions and aberrant cell growth [10–12].

1.2. Acetylation as a control mechanism for STAT proteins

While tyrosine phosphorylation critically controls the biological functions of STATs [8,12–14], reversible lysine acetylation has been revealed as an additional regulator of STAT activity [14–16] (Fig. 1 and Table 1). Two opposing enzymatic activities control the acetylation of lysine residues [16–18]. These are the histone acetyltransferases (HATs) and the histone deacetylases/sirtuins (HDACs/SIRTs). Since these enzymes target proteins in general they can also be called protein- or lysine-acetyltransferases/deacetylases [19]. HATs use acetyl-CoA to transfer the acetyl group and deacetylases use Zn^{2+} (HDACs) or NAD^{+} (SIRTs) as cofactors to deacetylate ε-N-acetylated lysine residues [20,21]. The first group of deacetylases falls into different classes [17,18,21]. In higher eukaryotes, HDACs are classified on the basis of their yeast counterparts. These are the ubiquitously expressed class I HDACs (HDACs1,-2,-3,-8); class IIa and IIb HDACs (HDACs4,-5,-7,-9 and HDAC6,-10) whose expression is restricted to certain cell types; and HDAC11 which belongs to class IV. SIRTs1-7, mammalian orthologues of *Saccharomyces cerevisiae* Sir2, comprise class III [17,18,20–22] (details on the structures and functions of these enzymes are provided in these references). These proteins can regulate gene expression and signaling as well as metabolic and genotoxic stress responses. Hence, they ultimately determine homeostasis and environmental adaptation [6,17,21,22].

Histone deacetylase inhibitors (HDACi) are used in ongoing clinical trials and have shown some success in the treatment of cancers, autoimmunity and for transplantations [23–25]. Due to different mechanisms of catalysis, SIRTs are not inhibited by HDACi and HDACs are not targeted by SIRT-inhibitors (SIRTi). Moreover, most HDACi preferentially inhibit certain HDACs and the benzamides and fatty acid-derived HDACi are even selective for class I HDACs. Compounds blocking HDAC classes I, II, IV are often called pan-HDACi [17,20–22,26]. A broad range of commercially available HDACi and isotype-selective RNAi tools facilitate a comprehensive understanding about the deacetylase-dependent

Table 1
STATs are acetylated on lysine residues and regulated by this posttranslational modification.

	Acetylation	Impact of acetylation on signaling	impact of acetylation on phosphorylation	Quality of acetylation on target genes	References
STAT1	↗	↗	↗	Inhibitory	[91]
	↗	↗	↗	Inhibitory	[90]
	↗	↗	↗	Inhibitory	[86]
	∅	Not tested	∅	Not tested	[104]
	↗	↗	↗	Inhibitory	[88]
	↗	Not tested	Not tested	Inhibitory	[41]
	↗	↗	Not tested	Not tested	[89]
	↗	Not tested	Not tested	Inhibitory	[87]
	Not tested	↗	Not tested	Inhibitory	[94]
	↗	Not tested	Not tested	Inhibitory	[37]
	Not tested	↗	↗	Inhibitory	[25]
	Not tested	↗	Not tested	Inhibitory	[92]
	Not tested	↗	Not tested	Inhibitory	[96]
	Not tested	↗	↗	Inhibitory	[73,95]
	Not tested	↗	Not tested	Inhibitory	[142]
	Not tested	↗	Not tested	Inhibitory	[97]
STAT2	↗	↗	Not tested	Positive	[89]
STAT3	↗	↗	∅/Not tested	Positive	[47,48]
	↗	↗	↗	Positive	[53]
	↗	↗	↗	Positve	[55]
	↗	↗	↗	Inhibitory	[72]
	↗	↗	∅	Positive	[62]
STAT5a	↗	↗	Not tested	Positive	[110]
STAT5b	↗	↗	Not tested	Positive	[51]
STAT6	↗	↗	Not tested	Positive	[30]

∅ negative result; ↗ positive results.

regulation of STAT proteins. Effects of HATs on STATs are usually tested by overexpression and knock-out or RNAi-based strategies. Deacetylation of STATs by HDACs1,-2,-3,-4 and SIRT1 and acetylation of STATs by CBP, p300, GCN5 and PCAF has been reported (Table 2).

JAK-STAT signaling is a paradigm for a pathway that generates loco-temporal dynamics *via* posttranslational modifications. This review presents an overview on STAT acetylation, the crosstalk of acetylation with other posttranslational modifications, the enzymes modulating STAT acetylation–deacetylation cycles and their biological consequences. We also discuss pharmacological strategies exploiting STAT de/acetylation. We summarize the reports on STAT acetylation chronologically.

2. Acetylation of STAT6

The interleukins IL4 and IL13 activate STAT6 which is immunologically relevant. STAT6 regulates type 2 T helper lymphocyte (Th2) differentiation and plays a role for asthma

Table 2
Putative acetylation sites of STATs and involved HATs/HDACs.

STAT protein	putative acetylation site	HAT	HDAC(s)	inducers	References
STAT1	K410, K413	CBP	HDACs1,-2,-3,-4	IFNα, IFNγ HDACi Cisplatin Calyculin/Pervanadate	[37,41,43,86–91]
	K679	?	?	?	[51]
	K173	?	?	?	[43]
STAT2	K182, K184, K194, K 197, K384, K390, K415, K419, K592	CBP, p300	?	IFNα	[89]
	K158, K384	?	?	?	[43]
	K375	?	?	?	[42,43,121]
STAT3	K685	CBP, p300	HDACs1,-2,-3 SIRT1	OSM, IFNα	[40,47,48,60,62]
	K49, K87	p300	HDAC1	IL6	[53–55]
	K679, K707, K709	CBP, p300	SIRT1	IL6, OSM, diet, SIRTi, SIRT activator	[60]
STAT4	K691	?	?	?	[51]
STAT5a	K84, K384	?	?	?	[43]
	K696	p300	?	IL7/FLT3L	[42,110]
STAT5b	K701	?	?	HDACi	[42,121]
	K359, K694, K701	CBP, p300, GCN5, PCAF	?	Prolactin IL7/FLT3L	[51,110]
	K84, K384	?	?	?	[43]
STAT6	?	CBP, p300	?	IL4	[30]
	K636	?	?	?	[51]
	K129*, K619*, K621*	?	?	?	[43]

See text for abbreviations; Table 2 lists data collected with mass spectrometry and point mutants (?: unknown; *: study tested the murine protein).

and other inflammatory lung diseases [27,28]. Furthermore, constitutively activated STAT6 is associated with lymphomas and leukemias [29]. In 2001, STAT6 became the first STAT family member reported to undergo lysine acetylation [30]. Analyzing IL4-treated A549 lung cancer cells, it became apparent that IL4 induces acetylation of STAT6 and that tagging of STAT6 with this posttranslational modification requires tyrosine kinase activity. Acetylation of STAT6 followed its tyrosine phosphorylation and the delayed acetylation was accompanied by histone H3 acetylation, induction of reticulocyte-type 15-lipoxygenase-1 (15-LOX-1) expression, and binding of STAT6 to the *15-LOX-1* promoter. The authors proposed from these data that non-acetylated histones at the *15-LOX-1* promoter prevent its induction by the rapidly phosphorylated STAT6 and that a time-delayed histone acetylation allows binding of acetylated STAT6 to induce 15-LOX-1 expression [30]. IL4 was furthermore found to globally augment the HAT activities of CBP and p300 [30].

Experiments with the viral protein E1A, which inhibits CBP, suggest that CBP is relevant for acetylation of STAT6 [30]. However, E1A can cause global relocalization of p300/CBP on promoters and alters the acetylation of histone H3 [18,31]. It was found that sodium butyrate blocks class I HDACs [17,20–22] and increases 15-LOX-1 expression. However, this experiment does not clarify whether this HDACi altered acetylation-dependent chromatin accessibility or STAT6 deacetylation directly. One could speculate that HDAC1 has an impact on the acetylation of STAT6, because a T cell-specific loss of HDAC1 promotes Th2 cytokine production and enhances allergic airway inflammation *in vivo*. Furthermore, HDAC1 binds the *IL4* gene to control the inflammatory response [32]. Since HDAC2 also plays a critical role in lung inflammation including asthma, chronic obstructive pulmonary disease and lung cancer [20] this class I HDAC may also deacetylate STAT6. Future experiments are necessary to test these ideas. Independent of above noted limitations, Shankaranarayanan and colleagues were the first showing that a STAT protein can become acetylated and they suggested a molecular mechanism for how acetylation might affect STAT6-dependent gene expression.

STAT6 contributes actively to the oncogenesis of lymphomas and leukemias in humans and rodent models [29]. Studies with HDACi argue that acetylation can correct aberrant STAT6 functions in Hodgkin lymphoma cells. For example, the clinically approved pan-HDACi SAHA (vorinostat) halts the growth of such cells. SAHA blocks STAT6 phosphorylation, decreases *STAT6* mRNA levels, and alters cytokine and chemokine secretion patterns in dendritic cells relevant for immunological surveillance [33]. It will be interesting to see whether these alterations correlate with STAT6 acetylation.

This also holds true for other drugs modulating phosphorylation of STAT6. An example is the active metabolite of leflunomide (teriflunomide, A771726) which is able to overcome resistance of chronic lymphocytic leukemia cells to the chemotherapeutically relevant anti-metabolite fludarabine [34]. While fludarabine blocks STAT1 [35], A771726 inhibits mitochondrial dihydroorotate dehydrogenase required for nucleotide metabolism and additionally reduces phosphorylation of STAT6 and STAT3. Together with the transcription factor NF-κB these STATs promote expression of anti-apoptotic factors (*e.g.* BCL-XL and MCL1) [34]. Beyond the question whether leflunomide and HDACi affect acetylation of STAT6, they may alter the STAT6/NF-κB interplay. This idea is based on the acetylation-dependent interactions between STAT1 and STAT3 with NF-κB, which itself is a most relevant factor for immunological functions and cancer development [10,36–41].

The biological consequences of site-specific lysine acetylation of STAT6 are unclear at present, but two sources state that human and murine STAT6 are acetylated at N- and C-terminal residues [42,43] (Fig. 1 and Table 2). Although identified most early, STAT6 acetylation is still a field with several open questions.

3. Acetylation of STAT3

Cytokines, growth factors, and oncoproteins induce phosphorylation of STAT3 on tyrosine-705 (Y705) and serine-727 (S727). Target genes of STAT3 are anti-apoptotic and growth-promoting, which renders STAT3 an oncogenic driver and a valid target for cancer therapy [44–46]. Cytokine-dependent acetylation of STAT3 at the C-terminal lysine-685 (K685) was reported in 2005 by two independent groups [47,48]. Both show that STAT3 undergoes acetylation in various adherent cancer-derived cell lines (Cervix, HeLa; liver, HepG2; breast, MCF7; embryonal kidney, 293T cells) that were treated with the related cytokines IL6 or OSM and with the class I interferon IFNα. The HATs p300 and CBP and HDACs1,-2,-3 were found to modulate acetylation of STAT3 [47,48] (Table 2).

Acetylated lysine residues can be found by mutagenesis scanning that replaces lysine residues and by mass spectrometry approaches [42,43,49,50]. A common way to subsequently analyze the role of acetylation is to introduce mutations mimicking or preventing acetylation. Exchange of lysine residues by glutamine (K → Q) imitates acetylation and mutations to arginine (K → R) prevent acetylation due to the mesomerically stabilized guanidine group of the arginine side chain [14]. The above mentioned studies by Wang et al. and Yuan et al. [47,48] found that disabling acetylation of STAT3 at K685 antagonizes dimerization, nuclear translocation, DNA binding, and gene expression. Roles of STAT3 acetylation became particularly evident in PC3 prostate cancer cells ($STAT3^{-/-}$ and $STAT5^{-/-}$) reconstituted with wild-type STAT3 or its K685 → R mutant ($STAT3^{K685R}$) [48] (Fig. 2). Notably, Yuan and colleagues could exclude that the inability of $STAT3^{K685R}$ to form dimers and activate transcription for cell proliferation relies on reduced tyrosine or serine phosphorylation [48].

The question remains how acetylation of STAT3 at K685, a residue residing on the external surface of the SH2 domain, affects signaling. A possible explanation, provided by Chin and colleagues, relies on the observation that K685 forms hydrogen bonds with two phenylalanine residues located in the hydrophobic core of the SH2 domain and this may prevent conformational changes necessary for the formation of the cytokine-induced transcriptionally active STAT3 dimer. In this scenario, acetylation blocks this interaction and thereby promotes STAT3 dimerization [48,51]. It will be interesting to see whether dimerization-incompetent STAT3 might be dephosphorylated more rapidly and if a $STAT3^{K685Q}$ variant, which mimics acetylation at this site, can cause a hyperactive phenotype resembling constitutively acetylated STAT3. If this is the case, there might even be tumor cells that carry this mutation as an oncogenic principle.

While K685 was found to be an acetylated site, K707 and K709 were excluded [47] and p300-, CBP-, and OSM-induced acetylation of STAT3 was reported to occur exquisitely at K685 [48]. However, $STAT3^{K685R}$ and $STAT3^{K685Q}$ were still slightly acetylated after co-transfection of p300 in the study by Wang and colleagues. It was speculated that residual acetylation of these mutants dimerizing with endogenous STAT3 caused the remaining acetylation signal [47]. In general, experiments involving STATs and mutants thereof are carried out best in stably reconstituted null backgrounds, *e.g.* PC3 cells [48], U3A and U6A cells [3], or STAT1/STAT3 knock-out cells [52].

Interestingly, STAT3 also has biologically relevant acetylation sites beyond K685. HDAC1-/p300-regulated acetylation sites were detected in the STAT3 NTD by Brasier and co-workers [53–55]. They reported an IL6-induced acetylation of the STAT3 residues K49/K87. Acetylation of these sites contributes positively to STAT3 signaling *via* recruiting p300 and stabilizing the enhanceosome (Fig. 2). This allows, *e.g.* transcription of monocyte chemotactic protein-1 in THP1 leukemia cells and human angiotensinogen in HepG2 hepatocellular carcinoma cells [53–55]. Nonetheless,

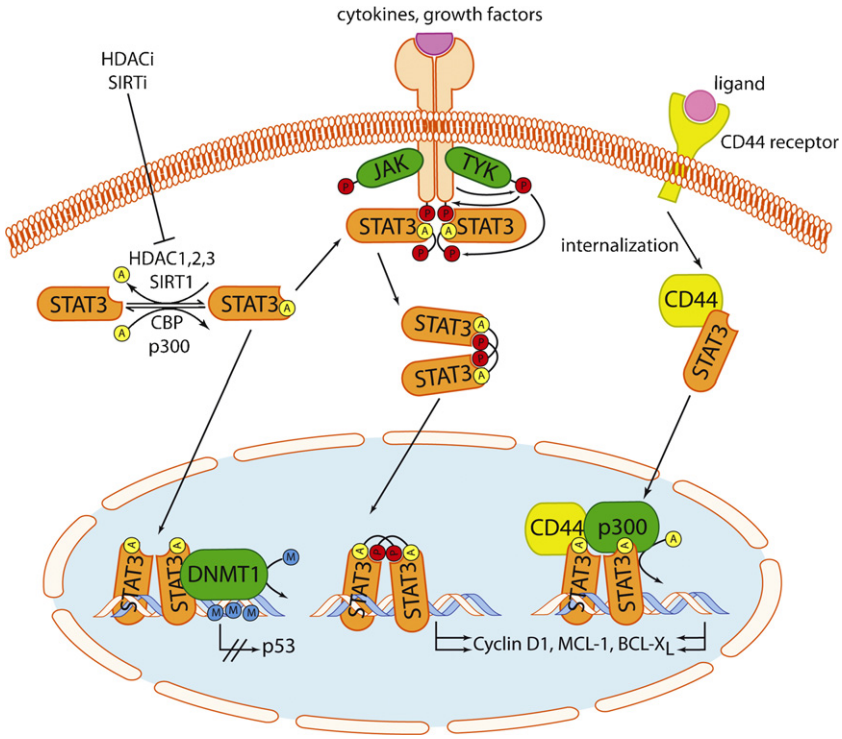

Fig. 2. Regulation of STAT3 acetylation by the cytokines IL6 and OSM, and by the glycoprotein CD44. Various signaling pathways allow acetylation of STAT3 and most reports suggest a positive role of acetylation on STAT3 signaling. IFNα and the related cytokines IL6 and OSM induce tyrosine phosphorylation and p300-dependent lysine acetylation of STAT3. The glycoprotein CD44 causes acetylation of STAT3 and tyrosine phosphorylation-independent induction of pro-survival STAT3 target genes.

knocking down HDAC1 leads to nuclear accumulation of STAT3 but has no effect on tyrosine phosphorylation of STAT3 [55] (Table 1). A phosphorylation-independent function of STAT3 acetylation is also evidenced by the observation that IL6, the pan-HDACi trichostatin A (TSA), and the SIRTi nicotinamide [17,23,24] promote nuclear accumulation of STAT3 in HepG2 cells [47].

Additional levels of complexity operate on STAT3 acetylation. For example, STAT3 acetylation can be induced cytokine- and growth factor-independently in cancer-derived cells (H1299, lung; HT29, HCT116, colon; AZ521/CD44, gastric, CD44 reconstituted) [56]. Upon ligation, the type I transmembrane glycoprotein CD44 (a cell surface glycoprotein and hyaluronan receptor) is internalized. *Via* its C-terminus CD44 interacts with the STAT3 NTD and facilitates its binding to p300. Subsequently, STAT3 becomes acetylated at K685 and activates the *Cyclin D1* promoter to accelerate tumor cell proliferation (Fig. 2). Of note, these processes are independent of tyrosine phosphorylation of STAT3. In contrast to STAT3^{K685R}, a STAT3^{Y705F} mutant becomes acetylated by p300 after CD44 ligation and acetylated STAT3^{Y705F} interacts with CD44 and other STAT3 molecules [56]. Whether the basal phosphorylation of STAT3 in H1299 cells is relevant for this mechanism is unclear at present and requires further experiments (*e.g.* with drugs blocking phosphorylation or reconstitution experiments in STAT3$^{-/-}$ backgrounds). CD44 has been implicated to be enriched and hence a marker for tumor-/cancer-initiating cells in solid and hematopoietic malignancies. Such stem cells significantly contribute to oncogenesis as they have properties of self-renewal and apoptosis resistance. They also sustain the tumor niche and epithelial–mesenchymal transition for metastasis [57,58]. Like CD44, STAT3 is a driving force of tumorigenesis [44–46,59]. The fact that CD44 transmits signals to STAT3 and evokes its acetylation [56] may integrate their pro-tumorigenic effects.

While already HDACs1,-2,-3 (HDAC8 was not tested) were found to act on acetylated STAT3 [47,48,54,55], a study analyzing a mouse model of feeding and fasting conditions revealed that SIRT1, and not HDAC1 or HDAC3, deacetylates STAT3 at K685. It was noted that SIRT1 binds STAT3 directly *via* its DBD, and that SIRT1-mediated deacetylation of STAT3 reduces its phosphorylation at Y705 [60]. Remarkably, SIRT1 is metabolically connected with STAT3 in the regulation of gluconeogenesis *versus* glycolysis. Under low caloric conditions NAD^+ is increased and activated SIRT1 inhibits the transcriptional activity of STAT3. Consequently, the STAT3-mediated repression of gluconeogenic target genes is relieved. These genes are controlled by the peroxisome proliferator-activated receptor gamma, coactivator 1 alpha which is activated by SIRT1-mediated deacetylation. Putative effects of SIRT1, SIRTi or SIRT activators on phosphorylation of JAK2 could be excluded which argues for acetylation as the primary regulator of SIRT1-dependent STAT3 signaling in human cells (293T and A2058 melanoma) and in primary and transformed murine cells [60]. The study by Nie et al. furthermore showed that STAT3^{Y705F} can undergo acetylation in response to OSM treatment which argues that acetylation of STAT3 occurs independently of tyrosine phosphorylation. By tandem mass spectrometry analysis, Nie and colleagues identified K679, which is highly conserved within the STAT family, as well as K707 and K709 as novel acetylated sites in STAT3 (Fig. 1). These are evolutionally conserved among mammalian STATs and are close to Y705 of STAT3 [60]. Unexpectedly and in contrast to the above mentioned studies [47,48], the study by Nie and co-workers [53–55] found only a minor reduction of acetylation of STAT3^{K685R}. They further noted that STAT3 K49/87R mutations have no effect on phosphorylation of STAT3 at Y705, and they show that TSA does not evoke acetylation of STAT3. Arginine substitution of all four lysines (K679/685/707/709 → R) reduced STAT3 phosphorylation far more strongly than the K685R mutant alone which indicates a regulatory importance of all acetylation sites found [60].

SIRT1 was also found to antagonize IL22-dependent STAT3 signaling in keratinocytes [61]. This regulation appears relevant for patients suffering from psoriasis as IFNγ decreases expression of

SIRT1 in keratinocytes of such patients and promotes basal STAT3 acetylation. Congruently, these cells react more sensitively to IL22 which induces STAT3-dependent psoriatic gene expression [61].

Another recent study showed that acetylated STAT3 attracts the cellular methylation machinery [62] (Fig. 2). In comparison to healthy tissue, STAT3 acetylated at K685 was found to be strikingly increased in melanomas, colon cancers and triple-negative breast cancers (basal-like phenotype; lacks estrogen receptor (ER), progesterone receptor and epidermal growth factor receptor-2). Overexpression of $STAT3^{K685R}$ in A2058 melanoma cells and in murine embryonic fibroblasts (MEFs) and a knock-in of $STAT3^{K685R}$ at the wild-type STAT3 locus in HCT116 colon cancer cells reduced promoter methylation of tumor suppressor genes including *STAT1*, *p53*, the STAT inhibitors *SOCS3* and *SHP1*, and the *cyclin-dependent kinase inhibitor 2A*, (*CDKN2A*, *p16* ^{Ink4A}) [62]. Mechanistically, acetylated STAT3 maintains expression of the DNA methyltransferase DNMT1 and recruits this enzyme to the above named tumor suppressor genes in cells and in mice. Acetylation-dependent formation of the STAT3-DNMT1 complex was found to be sensitive to TSA, the HAT inhibitor anacardic acid, and the SIRT activator resveratrol. Remarkably, reduction of acetylated STAT3 by resveratrol in triple-negative MDA-MB468 breast cancer cells and in M223 melanoma cells allowed demethylation and activation of the *ERα* gene. This induction of ERα sensitized tumor cells to the anti-estrogen tamoxifen [62]. Congruent with these data a study using triple-negative MDA-MB-231 breast cancer cells found that SIRT1 inhibition enhances silencing effects of DNMT1 on the *ERα* gene [63]. On the one hand, such findings render acetylated STAT3 and its effect on DNA methylation as valid targets for cancer therapy; on the other they also raise caution about the use of SIRTi.

In general, overactive STAT3 as well as overexpression of HDACs are hallmarks of cancers and both seem not compatible with the therapeutically exploited anti-tumor effects of HDACi [10,20,23,24,46,59]. Furthermore, constitutively active STATs (STAT1, STAT3, STAT5) are biomarkers predicting resistance of cutaneous T-cell lymphoma cells to SAHA [64]. One of the most potent pan-HDACi, the hydroxamic acid LBH589 (panobinostat), suppresses STAT3 phosphorylation in lung cancer cells with mutant epidermal growth factor [65]. About 2 million people suffer from lung cancer and it is promising that other studies also noted that HDACi reduce the proliferation of lung cancer cells [24,66–70]. HDACi also block STAT3 phosphorylation in normal cells, *e.g.* TSA abolishes phosphorylation of STAT3 but not STAT3 levels *per se* in renal fibroblasts. This observation might have implications for the treatment of renal fibrosis [71]. Moreover, poor prognosis diffuse large B cell lymphomas have constitutively active STAT3 and overexpression of HDAC3. Interestingly, LBH589 and a mere knock-down of HDAC3 are both cytotoxic to such cells. LBH589 increases acetylation of STAT3 at K685 and diminishes STAT3 phosphorylation at Y705. Accordingly, the amount of nuclear STAT3 and expression of the anti-apoptotic STAT3 target MCL1 become reduced [72]. Although these data contradict the positive role of acetylation on STAT3 phosphorylation and dimerization, they are in agreement with an increasingly large set of data that confirms HDACi as inhibitors of tumor growth [24,66–70]. Apparently, molecular mechanisms beyond direct acetylation of STAT3 appear more relevant for STAT3 signaling in clinically relevant contexts. A potential mechanism may rely on the inhibition of IFNγ-dependent JAK1 phosphorylation in RAS-deleted colon cancer cells when they are treated for extended time periods with TSA, SAHA, or butyrate [73]. Whether or not these mechanisms operate in other systems is an interesting question that remains to be clarified.

Additional effects of HDACi might, *e.g.* rely on effects on protein stability, alterations of chromatin structure, or on time-delayed processes including antagonistic effects, hormesis, and adaptation [17,20,24]. It is furthermore possible that HDACi affect STAT3-NF-κB and STAT3-p53 interplays, which are well documented regulators of oncogenesis *in vitro* and *in vivo* [10,12,36–39,41,74–76]. Acetylation may also affect the ability of STAT3 to control lysosomal membrane permeabilization by inducing lysosomal proteases (cathepsin-B/-L) and by suppressing the endogenous cathepsin-inhibitor SPI2A. This process induces the apoptotic regression of the mammary gland after the lactation period [77]. One can also imagine that acetylation has an effect on further molecules controlling STAT3. For example, the cis–trans peptidyl-prolyl isomerase cyclophilins A and B are relevant for several levels of STAT3 activation and do not target STAT1 [78]. Likewise, it will be interesting to see whether acetylation of STAT3 affects non-genomic functions. For example, transcriptionally inactive serine phosphorylated STAT3 is targeted to mitochondria. This portion of STAT3 is thought to affect electron transport, oxidative phosphorylation, RAS-dependent cellular transformation, and cellular robustness *via* reduction of ROS and mitochondrial cytochrome-c [79,80].

Acetylated STAT3 also controls immunological functions. A recent study shows that inhibition of HDACs1/-2/-3 with MS-275 (entinostat) induces acetylation of STAT3 and reduces expression of the transcription factor FOXP3 [81]. It might be that recruitment of DNMT1 by acetylated STAT3 [62] contributes to this regulation. FOXP3 is necessary for the development of regulatory T cells which attenuate anti-tumor responses [81]. The observation that a specific inhibitor of STAT3 partially rescued the down-regulation of FOXP3 by MS-275 suggests that acetylated STAT3 attenuates FOXP3 expression. Thus, acetylation of STAT3 may enhance the efficacy of cancer immunotherapy. Such effects may generate benefits *in vivo*, independent of the induction of pro-survival genes by acetylated STAT3, and this may be a reason for the efficacy of HDACi in some clinical settings [22–24]. Fig. 2 summarizes some knowledge on the acetylation of STAT3. Acetylation of STAT3 and of STAT1 has so far been most thoroughly investigated and still gives reason for attention and surprise.

4. Acetylation of STAT1

Interferons (IFNs) activate STAT1, a pivotal regulator of cell fate, growth, innate and adaptive immunity [1–3,82]. STAT1 null mice are viable but show increased sensitivity to viral agents and carcinogens [12]. Accordingly, STAT1 can promote apoptosis and recent evidence argues for an anti-oncogenic role of STAT1 in mammary gland and liver [83,84]. Nonetheless, STAT1 can support leukemogenesis by protecting cancer cells from natural killer cells [85] and it can promote chemotherapy resistance [86].

Acetylation of STAT1 in melanoma cells exposed to HDACi and IFNα was first reported in 2006 by our groups [37]. Meanwhile it has become clear that IFNs, lipopolysaccharide, alloantigens, and cisplatin induce acetylation of STAT1 *in vivo*. Pre-treatment of cells with HDACi as well as acetylation of STAT1 (*e.g.* in cells pre-stimulated with IFN or HDACi) interferes with STAT1 tyrosine-701 and serine-727 phosphorylation [14,15,25,41,73,86–97]. As a result, treatment of cells with HDACi does not evoke nuclear translocation of STAT1 but attenuate STAT1 signaling. It should be kept in mind that an apparent discrepancy regarding the effects of HDACi treatment on STAT1 phosphorylation can be solved by respecting different incubation times. While short term stimulation with HDACi and cytokines does not alter STAT1 phosphorylation [96,97], extended stimulation or longer pre-stimulation with HDACi induces accumulation of inert STAT1 [25,73,86,88,95] (Table 1). Similar findings were made for STAT5 (see below).

IFNα and IFNγ induce STAT1 phosphorylation–acetylation cycles that involve the T cell protein tyrosine phosphatase (TCP45) and CBP which both undergo nuclear to cytoplasmic

translocation upon cytokine stimulation [51,88,89]. This sequence of acetylation following phosphorylation allows a restricted STAT1-dependent gene expression pulse. Acetylation of STAT1 appears to increase its affinity for TCP45, and STAT1 dephosphorylation might prevent excessive cytokine stimulation and cell death [12,14,86–88,90,91]. Since the presence of the very active TCP45 close to acetylated STAT1 molecules allows quick dephosphorylation of associated STATs, only a presumably small quantity of STAT1 has to complete a full phosphorylation–acetylation switch. Furthermore, there is a low but detectable basal acetylation of STAT1 in resting cells [14,86–88,90,91]. Albeit it is possible that this is an irrelevant fraction of STAT1, basal acetylation of STAT1 may act positively to promote STAT1 signaling during the initial, productive phase of cytokine stimulation.

Remarkably, while CBP catalyzes acetylation of STAT1 *in vitro* and *in vivo*, other tested HATs (p300, GCN5, PCAF) were not found to catalyze acetylation of STAT1 in 293T and HeLa cells [37,88,89,91]. These HATs though modulate STAT1-dependent gene expression by other pathways involving regulation of chromatin structure [37,88,89]. Class-specific HDACi and experiments using RNAi against HDAC1,-2,-3 revealed that these HDACs, which are often overrepresented in tumors, are key players in the antagonistic relationship between phosphorylation-dependent STAT1 signaling and STAT1 acetylation [14,15,25,73,88,91–97]. The class II HDAC family member HDAC4 can also deacetylate STAT1 [86]. The observation that many HDACs critically control STAT1 acetylation may indicate that several steps evolved to control this posttranslational modification (Table 2). Although SIRTs are reported to deacetylate STAT3, it is unknown if these enzymes have any effect on STAT1 acetylation and subsequent downstream signaling events.

Evidence for a STAT1 phosphorylation–acetylation switch *in vivo* was recently collected in a murine model. It was found that the cell cycle regulator $p16^{Ink4a}$ regulates STAT1 phosphorylation and acetylation in macrophages [90]. Consistent with the above results, increased acetylation of STAT1 in $p16^{Ink4a-/-}$ cells leads to reduced phosphorylation of STAT1 at Y701. Increased expression of CBP in such cells is a possible molecular explanation for increased STAT1 acetylation. Perhaps, this $p16^{Ink4a}$-dependent STAT1 acetylation provides a fail-safe mechanism against tumorigenesis, as a loss of the cell cycle inhibitor $p16^{Ink4a}$ might be compensated by STAT1 acetylation which blocks NF-κB-dependent anti-apoptotic gene expression [37,41,74–76]. STAT1 also interacts with the tumor suppressor p53 to promote apoptosis and senescence. Such circuits might also be ruled by acetylation [12,98]. For example, IFN-induced cellular senescence depends on acetylation on p53 at K320 and occurs only in fibroblasts lacking $p16^{Ink4a}$ [99,100]. These processes might be connected *via* HDAC2, since HDAC2 antagonizes STAT1 signaling [20,73,95] and deacetylates p53 at specifically K320 [101].

Acetylation-dependent inhibition of STAT1 phosphorylation equally determines the sensitivity of primary and cultured ovarian cancers towards cisplatin, a highly relevant chemotherapeutic agent [86]. Of note, acetylated STAT1 is detectable in platinum-sensitive but not in resistant cells from the same patients. HDAC4 was found to interact and deacetylate STAT1 to support cancer cell survival under genotoxic stress induced by cisplatin. These data suggest a pro-survival role for STAT1 and a possible explanation for the frequently observed overexpression of HDACs in cancer tissues. Obviously, CBP-dependent STAT1 phosphorylation-acetylation switches control several levels of biology [86] (Table 1). STATs are not the only proteins controlled by such switches. For example, p53 is subject to a PCAF-dependent K320 acetylation/S15 phosphorylation switch [14,86,88,90,91,98,102].

Structural requirements for acetylation of STAT1 have been investigated. Before becoming dephosphorylated STAT1 dimers undergo parallel to antiparallel conformational transitions. These involve the NTD, CC, and DBD and allow presentation of pY701 to TCP45. STAT1 lacking critical phenylalanines in its NTD fail to undergo these conformational changes and remains phosphorylated far longer than wild-type STAT1 [9]. A STAT1 mutant carrying K → Q exchanges at positions 410/413 mimics the acetylated STAT1 and can be used as a surrogate for the constitutively acetylated STAT1 (Fig. 1). Like STAT1 acetylated after stimulation with IFN, $STAT1^{K410Q,K413Q}$ attracts TCP45 and resists IFN-induced tyrosine phosphorylation [37,88,91]. A mass spectrometry approach using Jurkat T cells that were treated with phosphatase inhibitors (calyculin and pervanadate) confirmed that K410 and K413 can be acetylated [43]. K410 and K413 of STAT1 are in the surface-exposed DBD and bind DNA [9]. Their acetylation or mutation to glutamine residues may relax p-STAT1/DNA contacts to facilitate structural rearrangement presenting pY701 to TCP45 [1,9,88,91]. Moreover, K410/K413 reside within the putative STAT1 NLS/NES, but L407 and L409 appear most critical for importin binding and nuclear entry of phosphorylated STAT1 [7,14,103]. Since binding of TCP45 prevents re-stimulation of STAT1 and TCP45 associates more strongly with acetylated STAT1 [88,91], it is tempting to speculate that dimerization is a prerequisite for STAT1 acetylation and association with TCP45. While additional experiments are required to test this hypothesis, it is known that STAT1 acetylation requires phosphorylation and nuclear translocation [88,91]. Naturally occurring STAT1 mutants that show prolonged tyrosine phosphorylation exist. 12 autosomal dominant STAT1 mutant alleles associated with reduced clearance of the pathogen *Candida albicans* and subsequent development of chronic candidiasis were described. These mutants carry defects in their coiled-coil domains and are impaired in tyrosine dephosphorylation. They show an increase in STAT1-dependent cellular responses to all three IFN classes (IFNsα/β,-γ,-λ [IL28/IL29]) and to IL6/IL21, which predominantly activate STAT3. This hyperactivation of STATs impairs the proper development of T cells producing IL17A, IL17F, and IL22 typically seen in patients suffering from chronic candidiasis.

Physiologically relevant differences between STAT1 homo-*versus* heterodimers have also been noted [1,3] and these also appear to determine the biological outcome of STAT1 acetylation [91,104]. For example, STAT1 and STAT3 can reciprocally affect each other [52,105] and such crosstalk can partially restore the activities of pseudo-acetylated $STAT1^{K410Q/K413Q}$ [91]. This finding is reminiscent of STAT1-STAT2 heterodimers in which STAT2 can overcome the inability of $STAT1^{L407A}$ to undergo nuclear translocation [103]. Both processes require phosphorylation of the defective STAT1 and its heterodimer partner and suggest a "piggy-back" mechanism that complements defects of STAT1. This model could explain why different outcomes of STAT1 acetylation in response to type I/II IFNs were seen [88,91]. Interestingly, analogous mechanisms evolved for nuclear hormone receptor signaling. For example, of two half sites of receptor response elements only one has to be recognized perfectly by one molecule within a heterodimer complex to allow DNA binding [106].

Covalent attachment of a member of the small ubiquitin-related modifier (SUMO) family to lysine residues can alter protein functions. Tyrosine phosphorylation of STAT1 is prevented in a UBC9-STAT1 fusion [107]. Apparently, bulky SUMO adducts attached to STAT1 at K703 disables tyrosine phosphorylation at Y705. The impact of sumoylation on endogenous STAT1 though seems weak and it is difficult to imagine how a small portion of endogenous sumoylated STAT1 (∼1–5%) [108,109] affects the entire pool of STAT1 without involvement of other molecules. Curiously, effects caused by mutation of K703 are not necessarily linked to sumoylation. Mutation of the sumoylation consensus motif ΨKxE (Ψ, large hydrophobic; K, lysine; x, any; E, glutamic

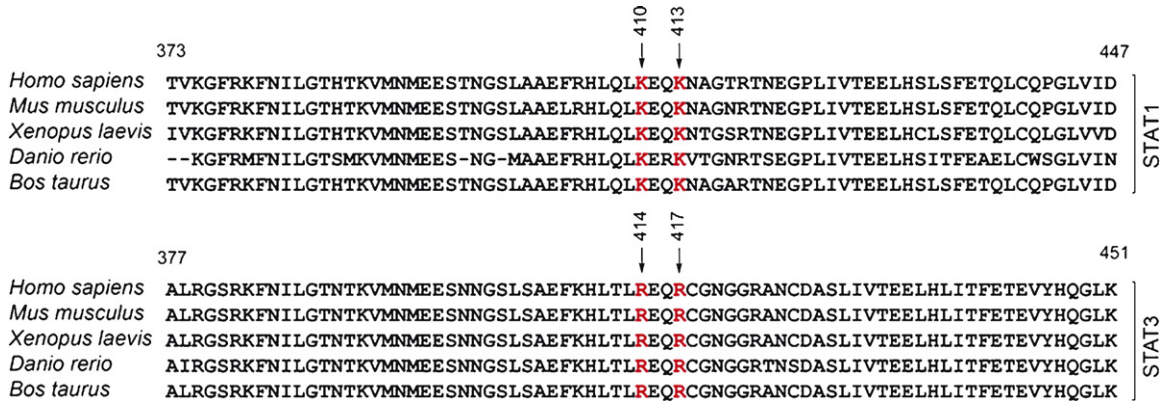

Fig. 3. STAT1/STAT3 sequence alignment. Sequence alignment analyses show that a particular difference between STAT1 and STAT3 in their DBDs is valid for different species, *e.g. Homo sapiens*, *Mus musculus*, *Bos taurus*, *Xenopus laevis*, *Danio rerio*. This also holds true for STAT2 [14].

acid) at K703 or E705 both block sumoylation but only replacement of the K703 causes prolonged tyrosine phosphorylation of STAT1 [109]. At present, it is unclear if K703 undergoes acetylation, methylation or any other type of posttranslational modification apart from sumoylation. The situation for STAT5 is though different as STAT5 undergoes a sumoylation/acetylation–phosphorylation switch [110] (see below). Curated mass spectrometry approach revealed that K410 (Jurkat T cells) and K173 (HCT-116 colon carcinoma cells) of STAT1 are acetylated (Fig. 1) and can also be ubiquitinylated [43]. Putative phosphorylation–ubiquitinylation switches may control the stability of STAT1.

Studies investigating HDACi and IFNα combinations found that they halt growth of melanomas [37] and other tumor cells more favorably than each agent alone [74–76]. At first sight, these data contradict inhibition of STAT1 signaling by HDACi. However, alternative pro-death mechanism in cells exposed to HDACi/IFN can be found; *e.g.* suppression of pro-survival signaling *via* NF-κB and increased expression of pro-apoptotic factors [37,41,74–76]. Endogenous HDACi (butyrate or sphingosine-1-phosphate) as well as pharmacological agents promote STAT1 acetylation and suppress IFN-/STAT1-dependent signaling [20,37,41,86–91]. The fact that HDACi promote STAT1 acetylation and impair STAT1-dependent IFN signaling might be a reason why they reduce anti-viral defense as well as inflammatory processes [14,15,25,73,92–97]. Counteracting unbalanced immunological functions and chronic inflammation linked to tumorigenesis is surely desired, but reduced anti-viral defense may impose a problem for patients treated with HDACi. Remarkably, this property of HDACi can though be exploited to increase the spread of oncolytic viruses and to promote virus-induced apoptosis of malignant cells *in vitro* and *in vivo* [93]. Hence, beyond their influence on epigenetic modifications of chromatin, blunting the cellular anti-viral response with HDACi could have a positive impact on therapeutics based on oncolytic viruses [22].

The above summarized data suggest that lysine residues within the STAT DBD tie in with the functional outcomes of acetylation within one cell. The NTD allowing heterodimerization appears particularly important in such scenarios. Interestingly, STAT orthologues from the slime mold *Dictyostelium discoideum* and the nematode *Caenorhabditis elegans* lack an amino-terminal oligomerization domain. This suggests that the primordial STAT lacked this domain and that it became accreted later in evolution to provide additional regulation within the context of cytokine responses [111]. Acetylation may confer different functionalities to closely related transcription factors that evolved from a common primordial STAT ancestor. Data from STA-1 (STAT orthologue) of *C. elegans* show that the DBD is not always involved in activation and nuclear accumulation of STATs [111]. This domain is a very necessary feature of JAK-STAT signaling in vertebrates. We speculate that at the same time it has evolved to antagonize STAT tyrosine phosphorylation within the context of increased acetylation. Here, the substantial differences between STAT1 and STAT3 regarding their acetylation-dependent regulation are striking. They are the most related STATs with 50% homology and both are substrates for TCP45 [4,14,112]. However, STAT3 cannot be acetylated at sites corresponding to STAT1 K410/K413, as these are arginines (R414 and R417) in STAT3 [14]. Sequence alignment analyses show that this particular difference between STAT1 and STAT3 is valid for different species (Fig. 3). STATs derived from a common primordial ancestor and acetylation has perhaps evolved as a rheostat for these molecules [4,14]. Domain swapping experiments might shed light on this question and on a putative transferability of STAT acetylation modules between STAT family members.

5. Acetylation of STAT2

Type I IFNs (IFNα/β) induce formation of the ISGF3 complex (STAT1-STAT2-interferon regulatory factor 9 [IRF9]) which is pivotal for the induction of anti-viral gene expression and innate cellular immunity [5,6]. Class I HDACs are important for acetylation-dependent IFN responses and viral defense [14,22,92]. One study found that CBP is recruited to the IFNα-stimulated IFNα-receptor (IFNAR) near its phosphorylated residues S364/S384 and acetylates K399 of IFNAR-2 [89]. Moreover, this work shows that STAT1, STAT2 and IRF9 are acetylated by CBP, and to a lesser extent by p300 in IFNα-stimulated HeLa and 293T cells. Experiments with STAT2 mutants revealed that acetylation of K390 located within the STAT2 DBD regulate formation of the ISGF3 complex and HDAC6-dependent anti-viral defense [89]. Whereas acetylation of STAT2 at exactly K390 appears required to allow association of STAT2 with IRF9, STAT2^{K390R} constitutively binds STAT1 but fails to recruit IRF9 in IFNα-treated cells. Since K390 is poorly conserved, Chin and colleagues speculate that it is an "evolutionary mistake requiring acetylation for correction" or a special feature pertinent to human cells only.

These data were collected with an elegant approach combining mass spectrometry and analysis of mutant molecules, and others have also shown acetylation of STAT2 (Fig. 1 and Table 2). It is though surprising that HDACi interfere with anti-viral gene expression [14,15,22,92,93,96,97]. For example, TSA blocks the virally induced IFNα/β promoters and IFN-dependent gene expression in murine L929 cells. TSA-dependent inhibition of ISGF3 and impaired nuclear accumulation of STAT2 appear as molecular mechanism for these observations [113]. Perhaps, acetylation of STAT1 and recruitment of TCP45 might be dominant

over positive effects of acetylation in cells with impaired HDAC or SIRT functions [88]. Moreover, a STAT1-STAT3 crosstalk exists in such cells [91] and treatment with IFNα together with TSA or silencing of HDAC1 and HDAC2 directs the IFNα response to STAT3 and away from the ISGF3 [114]. In addition, the above mentioned discrepancies can be explained by the two different settings, *i.e.* analysis of immediate IFN-induced STATs *versus* analysis of JAK-STAT signaling in cells with protein (hyper)-acetylation due to chemical or genetic HDAC inactivation.

6. Acetylation of STAT5

STAT5a and STAT5b are encoded by different genes and are often referred to as STAT5 [11]. Cytokines and growth factors activate STAT5 to regulate the proliferation, differentiation, and survival of cells. A lack of STAT5 causes insufficient hematopoietic differentiation, disturbed mammary development, and aberrant hepatocyte functions [11,115]. Moreover, conditional deletion of STAT5 in mice has shown that STAT5 acts as a mitogen and equally as transcriptional regulator that maintains quiescence during steady-state hematopoiesis [116]. While these data show that STAT5 is required to maintain homeostasis and proper development, aberrant constitutive activation STAT5 can occur in several types of leukemia and in solid tumors [115,117,118]. Accordingly, inhibition of overactive STAT5, *e.g. via* blocking the SH2 domain, can exert anti-leukemic effects [119].

Acetylation of STAT5b was first reported in 2010 by Ma and co-workers [51] and overexpressed CBP was found to catalyze acetylation of STAT5 effectively. In contrast to STAT1 for which acetylation by p300, GCN5, and PCAF could be ruled out [37,88,120], overexpression of p300 and to a lesser extent GCN5 and PCAF also enhance STAT5 acetylation [51] (Table 2). The precise molecular details establishing preferences of individual HATs for certain STATs remain to be identified. Acetylation-dependent dimerization of endogenous STAT5 was found in prolactin-treated T47D breast cancer cells. Mass spectrometry and site-specific antibodies revealed K359, K694, and K701 as acetylation sites of STAT5b. Furthermore, acetylation of STAT5b by CBP was found to occur independent of phosphorylation on K699 [51]. Acetylation of STAT5b^{K701} was also found in a large scale mass spectrometry approach [121]. STAT5 molecules carrying K → R exchanges at K694 revealed that acetylation of particularly this site is necessary for the prolactin-induced dimerization of endogenous STAT5 [51]. Whether K → Q exchanges mimicking acetylation at these sites create overactive STAT5 is an interesting, open question.

Like IFNα [88,120], prolactin induces nucleo-cytoplasmic shuttling of CBP to augment the cytoplasmic levels of CBP [51]. Thus, cytoplasmic accumulation of CBP can connect signaling from the membrane to the nucleus. While this is an interesting finding, it is also a puzzling one. It seems independent of JAK2 and a direct binding of CBP to the acetylated prolactin receptor is unlikely [51]. However, a role for other kinases and for the bromodomain of CBP, which recognizes acetylated lysines, cannot be excluded. All proteins are produced in the cytoplasm and access to the nucleus is a tightly regulated processes. It might be that IFNα and prolactin stop the nuclear import of CBP to augment its levels in the cytosol. CBP may recognize other acetylated molecules and/or acetylate proteins for nuclear import/export in the cytoplasm. Indeed, acetylation of importin-α has been identified as a control mechanism for nuclear import and export [17] and HDACi alter the cellular localization of CBP which binds transport proteins including importin-α [88,122].

Edward Yeh's lab recently provided evidence for a sumoylation/acetylation–phosphorylation switch controlling STAT5 in lymphocytes. They show that sumoylation and acetylation have opposing effects on the tyrosine phosphorylation of STAT5 [110]. A hierarchical cascade consisting of the E1 activating enzyme SAE1/SAE2, the E2 SUMO-conjugase UBC9, and E3 SUMO-ligases catalyzes sumoylation [123,124]. SUMO-specific proteases (SENPs, aka sentrin-specific proteases) deconjugate SUMO to establish highly dynamic sumoylation–desumoylation-cycles in cells. The SENP cysteine protease family consists of SENPs1-8 and certain SENPs are involved in tumorigenesis [125].

Analyzing a SENP1 knock-out model [126], it became clear to Van Nguyen and colleagues that knocking out STAT5 or SENP1 causes partially overlapping phenotypes and that STAT5 is highly sumoylated in B and T cells from SENP1$^{-/-}$ mice [110]. Biochemical analyses revealed that STAT5 is tagged with SUMO2/3, but not with SUMO1 in such cells. Accumulation of SUMO2-modified STAT5 in early lymphoid precursors reduced STAT5 acetylation. Induction of the anti-apoptotic protein BCL2 and lymphoid maturation were consistently blocked in SENP1$^{-/-}$ lymphocytes [110]. Furthermore it was found that phosphorylation at Y694 is necessary for sumoylation of STAT5a and that only nuclear STAT5 is sumoylated in primary B cells. STAT1 has a sumoylation site (K703) close to the phosphorylated tyrosine-701 [108,109] and comparison of the STAT5 protein sequences from *Homo sapiens*, *Mus musculus*, *and Rattus norvegicus* disclosed that two STAT5a/b lysine residues around the phosphorylated tyrosine 694 are conserved (K696/K703 in STAT5a and K701/K705 in STAT5b). Remarkably, mutation of K696 significantly reduced conjugation of ectopically expressed STAT5a with SUMO2 and acetylation of STAT5a K696R by p300 could not be detected with pan-anti-acetyllysine antibodies. This site hence appears as the major site for both, sumoylation and acetylation of STAT5a (Fig. 4). Furthermore, STAT5a K696 → R poorly induced the *β-casein* promoter. Interestingly, sumoylation and acetylation may have opposing effects on the tyrosine phosphorylation of STAT5 in T and B cells derived from murine hematopoietic stem cells. Endogenous STAT5b was found less acetylated at K701 in SENP1$^{-/-}$ lymphocytes when immunoblots were carried out with a STAT5b AcK701-specific antibody. These data suggest that a low percentage of sumoylated nuclear STAT5 affects the entire pool of STAT5 molecules in lymphocytes [110]. As sumoylation can sterically block phosphorylation only at one neighboring tyrosine

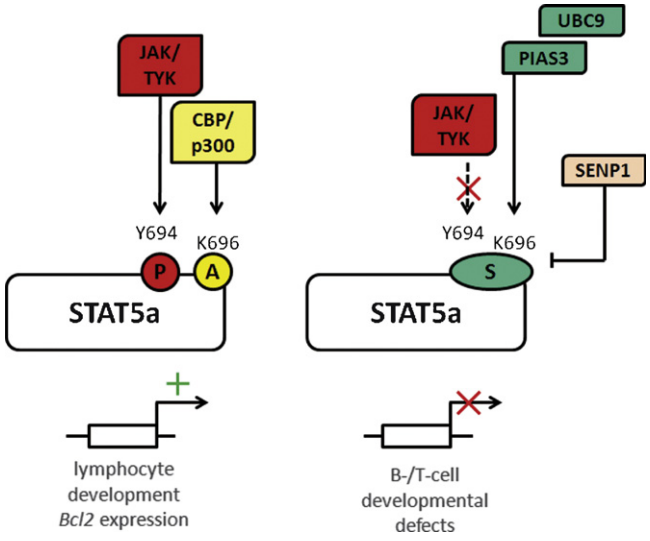

Fig. 4. A phosphorylation–acetylation/sumoylation switch regulates STAT5 signaling Acetylation (A) and sumoylation (S) of STAT5a and STAT5b were mapped close to Y694/Y699, respectively. Acetylation of these sites prevents sumoylation. Bulky SUMO2/3 residues can block tyrosine phosphorylation (P). STAT5a is shown as an example. This switch was investigated in the context of lymphoid development in SENP1 null mice.

phosphorylation site, additional unrecognized player(s) should be involved in the sumoylation-dependent control of STAT5.

While acetylation ties in with STAT5 function in cytokine-treated cells, it is unclear whether HDACi or SIRTi when given alone alter STAT5 acetylation. HDACi are used in ongoing clinical trials [22–24] and the HDACi TSA, SAHA, and butyrate were shown to block endogenous STAT5-dependent transcription initiation by preventing recruitment of the basal transcription machinery. These data were collected with IL3-/IL2-stimulated murine pro-B/T cells and it turned out that TSA had no effect on STAT5 phosphorylation or its nuclear translocation within an observation period of 1 hour [127]. Recent publications demonstrate that prolonged exposure of cells to HDACi suppresses phosphorylation and gene expression dependent on STAT5 in both, leukemias and solid tumor-derived cells [128–130] (and our unpublished data). An effect of HDACi on JAKs or phosphatases might be reasons for these findings. It is equally imaginable that acetylation may affect complex patterns upstream of STAT5, *e.g. via* the transcription factor MIZ1 that is linked to a SOCS1-dependent activation of JAKs [131,132]. The formation of tetramers and higher order structures also appears critical for leukemogenic effects of STAT5 [117,133]. Whether such structures contribute to the acetylation of STAT5 and other STATs might contribute additional exciting data on how STATs are controlled by acetylation.

7. Conclusions

The tumor suppressor p53 was the first non-histone protein described to be acetylated [120]. Comparison of the STAT3 acetylation level with the p53 acetylation level revealed that STAT3 is far less acetylated than p53 [47]. Nevertheless, lysine acetylation of STAT3 and other STATs appears as an important regulatory mechanism. It should be kept in mind that the amounts of a modified STAT might be comparably low, but such privileged pool may significantly affect other STATs *in trans* [7,35,88,91,103].

The current literature presents evidence on how acetylation determines functions of STATs and informs on how acetylation influences STAT signaling. The above described STAT1 phosphorylation–acetylation switch, the acetylation-dependent cascade activating STAT2, and the STAT5 sumoylation–acetylation/phosphorylation switch are examples for the complexity of acetylation-dependent control [88,89,110]. Perhaps it is also relevant to analyze phosphorylation beyond tyrosine phosphorylation, *e.g.* the newly discovered serine phosphorylation of STAT1 at S708 by the IκB kinase-ε. This posttranslational modification enhances anti-viral responses [134], as it inhibits STAT1 homodimerization and thereby promotes ISGF3-dependent anti-viral gene expression [135].

Whether "acetylation motifs" or modules which mimic acetylated residues (*i.e.* glutamine stretches) are transferable between the related STAT family members is also unclear. The transferability of "cassettes" such as *e.g.* the phosphorylation–acetylation or the acetylation–sumoylation switch can be tested in genetically defined models. It will also be interesting to see if splice variants of STATs are differentially acetylated. So far, STAT1 is the only example for which acetylation of both, STAT1α and STAT1β could be revealed [37,89]. If this ties in with different functions is though unknown.

Additional investigations will also find out whether phosphorylation of STATs by kinases other than JAKs is subject to control by acetylation. Kinases preferentially catalyzing tyrosine phosphorylation of STAT3 and STAT5 are for example c-KIT and FLT3-ITD. Interestingly, FLT3-ITD-positive human acute myeloid leukemia cells as well as murine and canine malignant mast cells harboring KIT mutations are very sensitive to HDACi [136,137]. These drugs might hence provide a therapeutic option for such diseases and STAT acetylation might serve as a pharmacological marker.

Different qualitative and quantitative cellular responses towards class I or II IFNs have been observed for decades, and cells are exposed to cytokine cocktails *in vivo* [2,5,11,15,91]. Despite its important role in immune responses and epigenetic control [138], least information regarding STAT acetylation is available for STAT4 (Fig. 1). There is one publication that describes its acetylation (Table 2) in a cursory manner. It is also surprising that hardly any data exist on a putative acetylation-dependent control of JAKs and phosphatases. Interestingly, not only CBP goes cytoplasmic after stimulation of cells with cytokines and HDACi [51,88,89,122] but JAK2 has equally been found in the nucleus [139] and may undergo cytokine/growth factor-dependent acetylation/deacetylation cycles.

Furthermore, it is interesting to find out whether non-mammalian STATs are controlled by acetylation and by regulatory switches involving acetylation. For example, *Drosophila melanogaster* mutants with reduced levels of unphosphorylated STAT show increased susceptibility to DNA damaging agents [140]. STATs and their acetylation may control genomic stability and reduction of STAT phosphorylation and expression by HDACi may be a reason for the frequently observed higher sensitivity of HDACi-treated cancer cells towards genotoxic chemotherapeutics [23,24,67,141].

The last decade presented acetylation as a novel posttranslational modification of STATs (Table 2). For some STATs the enzymes and biological consequences are rather clear, whereas other cases require further analyses (Table 1). JAK-STAT signaling and their posttranslational modifications will stimulate current and future generations of researchers.

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Martin Wieczorek graduated with a diploma degree in Biology at the University of Jena in the laboratory of T. Heinzel. During his undergraduate study he visited the Centre for Infection and Immunology (University of York) for a 6-month internship in P. Kayes' group. In his diploma thesis he worked on post-transcriptional regulation of the deacetylase SIRT1. After graduation he succeeded in T. Heinzel's group for a Ph.D. thesis. The main focus of his research project relies on post-translational modifications of nuclear hormone receptors and their subsequent biological implications for the receptor.

Torsten Ginter studied biochemistry at the University of Jena (Germany) and did his diploma thesis in T. Heinzel's lab at the CMB (Center for Molecular Biomedicine) in Jena. Afterwards he changed for his Ph.D. thesis into the group for Regulatory Protein Modifications led by O.H. Krämer at the CMB. Torsten investigates interferon-induced acetylation of STAT proteins and how this modification affects antiviral and inflammatory processes.

Peter Brand graduated with diploma in biology from Friedrich-Schiller-University Jena. During an exchange he worked on the involement of the histidin-rich glycoprotein on its angiogenic effects in tumor tissue in L. Claesson-Welshs group in the Rudbeck Laboratory in Uppsala, Sweden. Peter then joined the groups of S. Diekman and P. Hemmerich in the Leibniz-Institute for Age research to work on assembly dynamics of promyelocytic leukemia nuclear body in living cells for his graduation. Subsequently he started his Ph.D. in O.H. Krämer's group at the CMB to analyze epigenetic patterns in tumors and metastatic tissues.

Thorsten Heinzel obtained a Ph.D. in biochemistry from the Free University of Berlin based on his work at the Max-Planck-Institute for Molecular Genetics. He was a post-doctoral fellow for four years in the laboratory of M.G. Rosenfeld at the University of California, San Diego. Subsequently, he was a staff scientist at the German Cancer Research Center in Heidelberg. He then moved to the Biomedical Research Institute Georg-Speyer-Haus in Frankfurt/Main where he stayed as a group leader for 6 years. Currently, Dr. Heinzel is full professor and holds the chair of biochemistry at the Friedrich-Schiller-University Jena. He is a member of the Center of Molecular Biomedicine and Vice-Rector of Research of the Friedrich-Schiller-University. In 2006 he received the Cancer Research Award of the German Cancer Research Society (DKG). His primary focus of research is the role of protein acetylation in gene regulation.

Oliver H. Krämer studied biology and pharmacology at the Ruprecht-Karls University Heidelberg. He obtained a Ph.D. in biochemistry from the Johann-Wolfgang-Goethe University based on his work at the Georg-Speyer-Haus in Frankfurt/Main. He was a post-doctoral fellow in the laboratory of T. Heinzel at the Georg-Speyer-Haus and later on in the CMB. Three years ago, he has become a group leader at the CMB. Dr. Krämer is associate professor at the Friedrich-Schiller-University. His research focuses on acetylation-dependent signaling for cell survival and the proteasomal degradation of cancer-relevant molecules.

Manuskript 3: Acetylation of Endogenous STAT Proteins

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Corresponding Author	Family Name	Krämer
	Particle	
	Given Name	**Oliver H.**
	Suffix	
	Division	Center for Molecular Biomedicine (CMB)
	Organization	Department of Biochemistry, University of Jena
	Address	Hans-Knöll-Str. 2, 07745, Jena, Germany
	Email	Oliver.Kraemer@uni-jena.de
Author	Family Name	Ginter
	Particle	
	Given Name	**Torsten**
	Suffix	
	Division	Center for Molecular Biomedicine (CMB)
	Organization	Department of Biochemistry, University of Jena
	Address	Hans-Knöll-Str. 2, 07745, Jena, Germany
	Email	
Author	Family Name	Heinzel
	Particle	
	Given Name	**Thorsten**
	Suffix	
	Division	Center for Molecular Biomedicine (CMB)
	Organization	Department of Biochemistry, University of Jena
	Address	Hans-Knöll-Str. 2, 07745, Jena, Germany
	Email	

Abstract	Acetylation of signal transducer and activator of transcription (STAT) proteins has been recognized as a significant mechanism for the regulation of their cellular functions. Site-specific antibodies are available only for a minority of STATs. The detection of acetylated STATs by immunoprecipitation (IP) followed by western blot (WB) will be described in the following chapter. Defined conditions for cell lysis and IP will be elucidated on the basis of STAT1 acetylation.

Key words: (separated by '-')	Acetylation - HDACi - HAT - IFN - Immunoprecipitation - STAT - STAT1

Chapter 12

Acetylation of Endogenous STAT Proteins

Torsten Ginter, Thorsten Heinzel, and Oliver H. Krämer

Abstract

Acetylation of signal transducer and activator of transcription (STAT) proteins has been recognized as a significant mechanism for the regulation of their cellular functions. Site-specific antibodies are available only for a minority of STATs. The detection of acetylated STATs by immunoprecipitation (IP) followed by western blot (WB) will be described in the following chapter. Defined conditions for cell lysis and IP will be elucidated on the basis of STAT1 acetylation.

Key words: Acetylation, HDACi, HAT, IFN, Immunoprecipitation, STAT, STAT1

1. Introduction

Acetylation as a posttranslational modification (PTM) was initially discovered for histones (1). In addition to histones, a rising number of nonhistone proteins (e.g., STATs, p53, and NF-kB) are found to be regulated by acetylation (2, 3). The equilibrium of acetylation and deacetylation is very dynamic and mainly regulated by two enzyme families. Histone acetyltransferases (HATs) transfer acetyl groups from acetyl-CoA molecules to the ε-NH_2 groups of lysine side chains. The histone deacetylases (HDACs) catalyze the removal of the acetyl groups. HDACs need Zn^{2+} or nicotinamide (NAD^+) as cofactors (4). The Zn^{2+}-dependent HDACs are termed as the classical family and are divided in class I (HDAC 1,2,3,8), class II (HDAC 4,5,6,7,9,10), and class IV (HDAC 11). NAD^+-dependent HDACs are designated sirtuins (SIRTs) and comprise class III (SIRT 1–7). In contrast to HDACs, the classification of HATs is less clear (the interested reader is referred to the literature, e.g., (3)). HATs and HDACs are often deregulated in cancer (5), with aberrant protein levels detectable in many tumor types (6, 7). Researchers have developed HDAC inhibitors (HDACi), which could be promising drugs for chemotherapy (8).

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Since HDACs of the classical family share-related active sites, several pan-HDACi were found. Trichostatin A (TSA) a fungal antibiotic, suberoylanilide hydroxamic acid (SAHA), LAQ-824, and LBH589 are representatives of this group. Class I selective inhibitors like MS-275, valproic acid (VPA), and depsipeptide do exist (3), but strictly isoform-specific inhibitors still remain to be generated (9). Due to the need of NAD^+ as a cosubstrate for SIRTs, nicotinamide serves as a noncompetitive inhibitor for this class of enzymes (10). HDACi are essential tools for the detection of acetylated proteins and hence have to be a component of the cell lysis buffer (11).

Transcription factors of the STAT family play key roles for cell survival, differentiation, proliferation, and homeostasis. Cytokines bind to cognate receptors and activate Janus kinases (JAKs), which catalyze tyrosine phosphorylation of STATs at defined sites (12). Tyrosine phosphorylation of STATs allows the nuclear import of preformed cytosolic dimers (13, 14). These act as specific inducers of gene sets relevant for physiological processes (15).

Besides phosphorylation, acetylation has turned out as a PTM that crucially regulates STAT activity. In the last years all STATs, except for STAT4, have been positively tested for acetylation (Table 1). The consequences of this modification for signaling are diverse. For example, acetylation of STAT3 ties in with dimerization and transcriptional activation (16, 17). However, we and others showed that acetylation of STAT1 is inactivating, as it causes diminished phosphorylation (11, 18–24). It is accordingly accepted that acetylation blocks STAT1-dependent signaling and gene expression (22, 25–31).

Figure 1 shows an example for the detection of STAT1 acetylation in SK-Mel-37 cells after exposure to different treatments. Cytokine-induced acetylation of STAT1 occurs time-dependently (Fig. 2) and ensues the very rapidly induced and transient phosphorylation of STAT1 (18, 19, 22).

Table 1
Acetylation of STAT proteins; HAT, histone acetyltransferase

STAT protein	Lysine moieties required for acetylation	HAT	Tested stimulus	References
STAT1	K410, K413	CBP	IFNα, IFNγ HDACi Cisplatin	(11, 18–24)
STAT2	K390	CBP	IFNα	(23)
STAT3	K685	p300	OSM, IFNα	(16, 37–39)
	K49, K87	p300	IL6	(17, 38)
	K679, K707, K709	?	IL6	(38)
STAT5b	K359, K694, K701	CBP	Prolactin	(40)
STAT6	?	CBP/p300	IL4	(41)

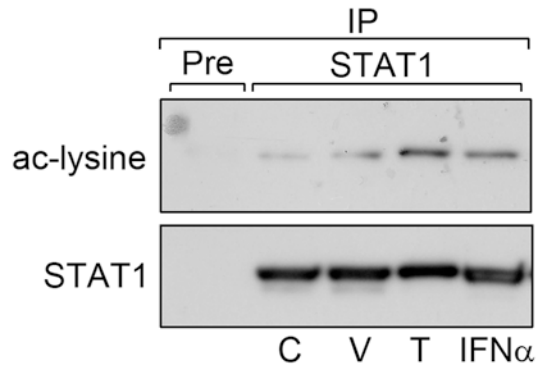

Fig. 1. SK-Mel-37 cells were treated with the HDACi VPA (V; 3 mM) and TSA (T; 100 nM) or the cytokine interferon alpha (IFNα; 1,000 U/ml) for 24 h. Subsequently, cells were immunoprecipitated under acetylation preserving conditions with anti-STAT1 antibody (Santa Cruz, sc-417). Acetylated STAT1 was detected using a pan-specific anti-acetylated lysine antibody (Cell Signaling, # 9441) for WB; C, untreated cells; IP, immunoprecipitation; Pre, pre-immune serum.

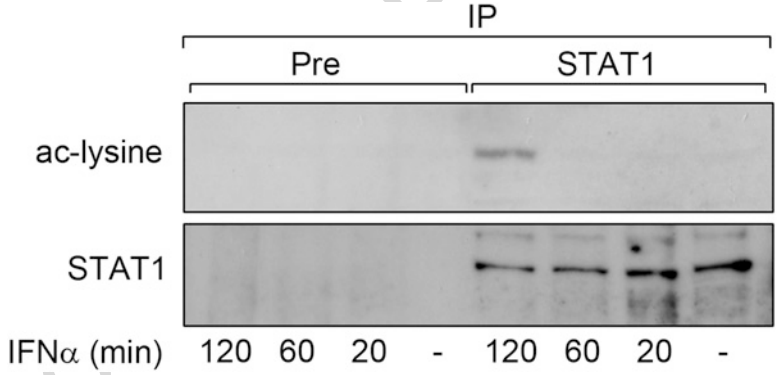

Fig. 2. SK-Mel-37 cells were stimulated with 1,000 U/ml IFNα for the indicated times. Whole cell lysates were immunoprecipitated with STAT1 antibody (Santa Cruz, sc-417) and probed for acetyl-lysine (Cell Signaling, # 9441) by WB analysis; IP, immunoprecipitation; Pre, pre-immune serum.

Several methods may be employed for the detection of acetylated proteins. The most convenient method is a WB analysis using a site-specific antibody. Unfortunately, such agents are still a rarity and their generation is time consuming and expensive. Alternatively, mass spectrometry with enriched acetylated peptides from trypsin-digested whole cell extracts could be performed. However, biologically important acetylation sites of low abundance will remain undetected, because of a high background of non-acetylated peptides (32). This situation is reminiscent of the PTM with SUMO, where only a small portion of protein needs to be modified to evoke important physiological processes (33). Furthermore mass spectrometry requires much technical equipment and expert

knowledge. Radioactive labeling of lysines with ^{14}C-acetyl-CoA is an alternative to immunodetection and applicable after in vivo labeling or enzymatic in vitro acetylation of recombinant proteins. The disadvantage of in vivo labeling can be high background signals of other acetylated proteins even upon performance of 1D/2D gel electrophoresis. In vitro labeling of recombinant proteins could be determined easily by scintillation counting (32, 34).

For in vivo analysis of acetylation, an IP of the protein of interest from whole cell lysate, followed by SDS polyacrylamide gel electrophoresis (SDS-PAGE) and WB analysis with pan-specific acetyl-lysine antibodies is often needed (Figs. 1 and 2). This method is most frequently used to analyze protein acetylation and will be described in this chapter using the example of endogenous STAT1 acetylation.

2. Materials

Materials and antibodies listed here are routinely used by our lab and many other groups. However, equipment from other providers should be equally useful.

2.1. Preparation of Whole Cell Extracts

1. RIPA lysis buffer: 0.1–1% sodium dodecyl sulfate (SDS) (w/v) (see Note 1), 1% sodium desoxycholate (w/v), 1% NP-40 (v/v), 50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1 mM EDTA. Freshly add HDACi (TSA and nicotinamide) as indicated (see Notes 2 and 3). For codetection of phosphorylation 1 mM sodium vanadate and 0.5 mM NaF has to be added.
2. Sonification: Branson Sonifier W250D amplitude 40% for 3 s ten times.
3. Dulbecco's Phosphate-Buffered Saline (PBS), e.g., from PAA.

2.2. Immunoprecipitation

1. Antibodies for IP and WB.
   (a) STAT1 (Santa Cruz; sc-346 [rabbit]).
   (b) STAT1 (Santa Cruz; sc-417 [mouse]).
   (c) Pan-specific acetyl-lysine (Cell Signaling; # 9441 [rabbit]).
   (d) Pan-specific acetyl-lysine (Cell Signaling; # 9681 [mouse]).
   (e) Acetyl-histone H3 (upstate; 06-599 [rabbit]).
   (f) Pre-immune serum (Santa Cruz; sc-2025 [mouse]).
   (g) Pre-immune serum (Santa Cruz; sc-2027 [rabbit]).
   (h) See Note 4.

2. Protein A Sepharose CL 4B and Protein G Sepharose 4 Fast Flow (GE-Healthcare): Follow the customer instructions to equilibrate and wash the slurry. Resuspend the Sepharose in RIPA buffer and mix protein A Sepharose/protein G Sepharose in a ratio 1:1 (see Note 5).
3. SDS-Laemmli (2×) loading buffer: 116 mM Tris–HCl pH 6.8, 1.4 M β-mercaptoethanol, 10% glycerol, 3.3% SDS (w/v), spatula tip Bromophenol blue.

2.3. SDS Polyacrylamide Gel Electrophoresis

1. Separating gel buffer: 1 M Tris–HCl pH 8.8.
2. Stacking gel buffer: 1 M Tris–HCl pH 6.8.
3. 20% SDS: 20% (w/v) aqueous solution.
4. 10% Ammonium persulfate (APS): 10% (w/v) aqueous solution.
5. Tetramethylethylenediamine (TEMED) (Sigma).
6. 30% Acrylamide/bisacrylamide 37.5/1 (Roth).
7. Butanol (Roth).
8. Mini Protean 3 system—casting stand with corresponding casting frames, combs, and glass plates (spacers included) (Bio-Rad).
9. SDS-Running buffer: 250 mM glycine, 25 mM Tris, 0.1% (w/v) SDS (see Note 6).
10. Protein ladder (Bio-Rad/ Jena Bioscience).

2.4. Protein Transfer—Western Blot

1. Polyvinylidene difluoride (PVDF) membrane (Millipore).
2. Whatman paper (3 M) (VWR Scientific).
3. Ethanol (Roth).
4. Transfer buffer: 250 mM glycine, 25 mM Tris, 0.1% (w/v) SDS, 20% ethanol (see Note 7).
5. Western transfer apparatus (Bio-Rad).

2.5. Antiacetyl-Lysine Detection

[AU2]

1. Antibodies see Subheading 2.2, item 1.
2. Nonfat dry milk (NFDM) (see Note 8).
3. PBS-T: 137 mM NaCl, 8 mM Na_2HPO_4, 2.7 mM KCl, 1.4 mM KH_2PO_4, adjust with HCl to pH 7.25, 0.05% Tween 20.
4. Enhanced Chemoluminecence (ECL) kit (Thermo scientific).
5. Stripping buffer: 62.5 mM Tris–HCl pH 6.8, 2% (w/v) SDS, add freshly 100 mM β-mercaptoethanol (β-ME).

3. Methods

3.1. Preparation of Cell Lysate

Acetylation as a PTM has a very transient nature. The fine-tuned equilibrium of this modification is regulated by the opposing enzymatic activities of HATs and HDACs. Moreover, several PTM can compete for the same lysine residue (3). The challenge is to preserve the acetylation during cell lysis, IP, and WB. Therefore, the experimentator has to take care that the lysis buffer is very stringent to disturb HDAC–substrate complexes required for deacetylation reactions. Additionally, HDACi are added to the buffers and all work steps are done on ice to decelerate remaining HDAC activities.

1. Human or murine cells are cultured in 90 mm dishes to approximately 90% confluence and are stimulated with an appropriate ligand (e.g., 1,000 U/ml IFNα for 3 h) or an HDACi. For each IP at least $3–4 \times 10^6$ cells are required.
2. Every following step is done on ice to slow down HDACs and prevent deacetylation. Before harvesting, cells are washed with ice-cold PBS, containing 200 nM TSA and 5 mM nicotinamide.
3. Cells are lysed immediately in the dish using 1 ml RIPA buffer (containing 1 µM TSA and 5 mM nicotinamide). Take a rubber policeman to scratch the cells from the bottom of the plate. The lysate should appear viscous.
4. Subsequently, the RIPA lysate is sonified directly to reduce viscosity.
5. A centrifugation step at $20,000 \times g$ for 5 min at 4°C is performed to remove cellular debris. It is recommended to directly use the fresh lysate for the IP. Alternatively it can be stored at −80°C or below.

3.2. Immunoprecipitation

The success of an IP depends on several factors. The most important is beyond all questions the reliability of the antibodies. In Subheading 2.2, item 1, antibodies for detection of acetyl-STAT1 are listed (see Note 9). It is necessary to exclude unspecific binding by the usage of a pre-immune serum, i.e., a mock-IP with the same lysate.

1. 500 µl of RIPA lysate are incubated with 1 µg of antibody (e.g., sc-417) and 40 µl of protein A/G sepharose (GE-healthcare) in a ratio 1:1 overnight at 4°C on a rotator. Sepharose slurry was equilibrated in RIPA buffer before use. 500 µl of the same lysate or a pool of several samples is incubated with pre-immune serum in parallel. In addition 5–10% lysate can be taken off for input control (see Note 10).

2. On the next day, IP samples are centrifuged at 5,000 × *g* for 1 min at 4°C. Supernatant is aspirated or could be alternatively transferred to a new Eppendorf tube for further analyses (e.g., depletion efficacy of the antibodies).
3. Afterwards the Sepharose beads are washed in 400 µl RIPA (containing freshly added 200 nM TSA and 5 mM nicotinamide) and are centrifuged as before. This step is repeated for three times.
4. Residual RIPA buffer is removed with a precision syringe and beads are boiled in 30 µl SDS-Laemmli (2×) for 5 min at 95°C. Spin the samples briefly down and do SDS-PAGE immediately.

3.3. SDS Polyacrylamide Gel Electrophoresis

1. Cast the 8% acrylamide (v/v) separating gel as stated in Table 2 (see Note 11). Polyacrylamide is built in a radical polymerization. Since APS is the radical former and TEMED the catalyst, both should be added lastly to the mixture. Promptly pour the composite into the assembled gel plate to 3/4 of the volume (see Note 12). Cover the gel surface with 200 µl butanol to ensure proper polymerization.
2. After approximately 30 min the separating gel is polymerized. Pour away the butanol and remove remaining butanol by washing twice with water. Carefully draw off residual water using filter paper. Cast the 5% stacking gel as indicated in Table 2 and quickly insert the comb.
3. Approximately 20 min later stacking gel should be polymerized and the comb could be removed carefully. To clean the slots rinsing with ddH_2O is done twice.

Table 2
Composition of separating and stacking gel

Stacking gel		Separating gel			
		8%	10%	12%	15%
Rotiphorese Gel30	415 µl	2 ml	2.5 ml	3 ml	3.8 ml
Stacking gel buffer	312 µl	–	–	–	–
Separating gel buffer	–	2.8 ml	2.8 ml	2.8 ml	2.8 ml
SDS 20% (w/v)	12.5 µl	37.5 µl	37.5 µl	37.5 µl	37.5 µl
H_2O	1.75 ml	2.6 ml	2.1 ml	1.6 ml	0.9 ml
APS 20% (w/v)	12.5 µl	37.5 µl	37.5 µl	37.5 µl	37.5 µl
TEMED	2.5 µl	5 µl	5 µl	5 µl	5 µl

4. The gel could be used directly for electrophoresis, but it is recommended to wrap the gel in wet paper and store it overnight at 4°C to ensure complete polymerization as this results in better separating capabilities (see Note 13).
5. Assemble the gel in an electrophoresis chamber (Bio-Rad) and fill up with SDS-Running buffer.
6. Use a Hamilton syringe to load the samples from Subheading 3.2, step 4 into the slots of the stacking gel and do not forget a protein ladder.
7. Subsequently, run the gel for approximately 1.5 h at 20–30 mA. For orientation the Bromophenol blue from the SDS-Laemmli will mark the running front of the gel.

3.4. Protein Transfer—Western Blot

For the analysis of negatively charged proteins (through SDS attachment) by WB, proteins are transferred by electrical field force to a PVDF membrane. Two major methods exist for protein transfer. Semidry blotting is sufficient for proteins smaller than 70 kDa, but for bigger proteins the transfer efficiency is often poor. An alternative is wet blotting, which provides even convincing transfer results for proteins bigger than 130 kDa. Figure 3 shows a schematic view of a wet blotting configuration.

1. A PVDF membrane (usually 6×9 cm) is equilibrated in ethanol for 1 min. Afterwards the membrane is incubated in transfer buffer for 10 min (see Note 14).
2. In the meantime soak two sponges and two Whatman papers (usually 6×9 cm) with transfer buffer and begin to assemble the sandwich (Fig. 3) in a tray filled with transfer buffer. Use a role (e.g., 15 ml tube) to remove air bubbles between the layers.
3. Take the polyacrylamide gel and cut off the stacking gel. The separating gel is briefly incubated in transfer buffer and then carefully placed onto the PVDF membrane. Avoid air bubbles

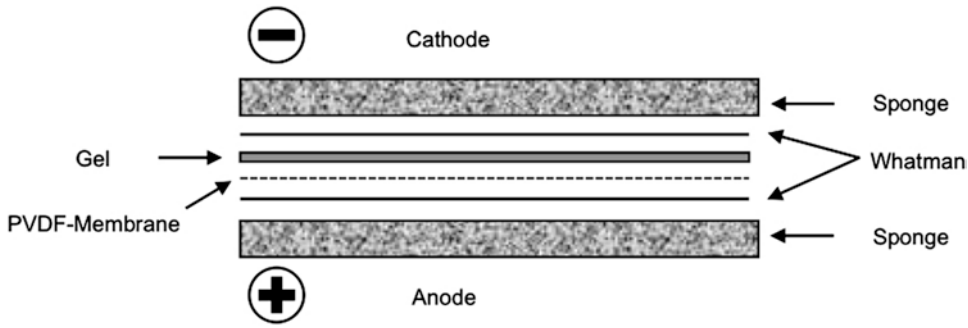

Fig. 3. Schematic configuration of a wet blotting protein transfer assembly.

between the layers, since this results in insufficient protein transfer.

4. Cover the gel with the second piece of Whatman and an additional sponge (Fig. 3).
5. Prepare a wet blot apparatus filled with transfer buffer and insert the assembled sandwich.
6. The transfer should run for 2 h at 150 mA per gel and 4°C (usually in a cold room).
7. Disassemble the apparatus and transfer the membrane to a tray filled with PBS-T. Incubate the membrane in PBS-T on a rocking platform for 5 min.

3.5. Antiacetyl-Lysine Detection

1. Incubate the PVDF membrane in PBS-T with 5% (w/v) nonfat dry milk (NFDM) for 1 h on a shaker to block unspecific binding sites on the membrane.
2. Add the membrane to a tray with PBS-T, 2% (w/v) NFDM, pan-acetyl-lysine antibody (e.g., 9441) diluted 1:1,000 and sodium azide 0.01% (w/v). Incubate over night at 4°C on a shaker (see Note 15).
3. Wash the membrane three times for 10 min in PBS-T on a shaker to reduce unbound antibodies and sodium azide.
4. Subsequently, incubation with the secondary antibody diluted 1:5,000 in PBS-T with 2% NFDM occurs for 1–2 h at room temperature.
5. Afterwards the membrane is washed three times for 10 min with PBS-T to remove unbound secondary antibodies.
6. The signal is detected by autoradiography using a Thermo scientific ECL kit as recommended by the manufacturer. In brief the kit contains two solutions, which are mixed in a ratio of 1:1. 1 ml of this mixture is dispensed homogeneously on the membrane. Put the membrane in a transparent plastic bag and wipe off excessive ECL solution. Maintaining some ECL solution may prevents fast substrate depletion and false-negative signals.
7. After detection of acetylation the identification of total STAT protein level is relevant. For that purpose wash the membrane briefly in PBS-T and afterwards incubate the membrane in stripping buffer for 1 h at room temperature on a shaker (see Note 16).
8. Wash the membrane several times with distilled water until the smell of β-ME has disappeared entirely. Incubate the membrane briefly in PBS-T and continue with steps 1–6.

4. Notes

1. Some antibodies are ineffective at a concentration of 1% SDS. If this is the case reduce the SDS concentration.
2. Please note that, certain protease inhibitors inactivate hydroxamic acid-derived HDACi and therefore can block their effects preserving acetylation (35).
3. It is preferable to prepare a TSA stock of 100 µM dissolved in DMSO and a 1 M aqueous solution of nicotinamide. Both stocks should be stored at −150°C. Before starting the experiment, it is recommended to test the biological activity of the HDACi stocks. This could be done by analyzing global histone acetylation (antibody 06-599). However, this is never a control for the preservation of protein acetylation during IP conditions.
4. Please note that certain antibodies, specifically select for non-acetylated proteins. They do not recognize the acetylated form because the epitopes are masked by acetylation. An example is PAb421, which only identifies non-acetylated forms of p53 (36).
5. Add sodium azide (very toxic) 0.01% (w/v) to prevent microbial contamination and BSA 0.05% (w/v) to diminish unspecific protein binding to the slurry (store at 4°C).
6. A stock of tenfold concentrated SDS Running buffer is preferable.
7. Many protocols recommend methanol, but ethanol serves the same purpose and is less harmful.
8. Some authors reported good results using Roti©-Block (Roth) instead of NFDM.
9. It is strongly recommended to use antibodies from different species for IP and WB, because this reduces background signal.
10. An input control is done to assure equal protein levels before the IP. Furthermore, to detect STAT1 acetylation an efficient IP is absolutely required. Thus, make sure that there is enrichment of protein compared to the input (five- to tenfold).
11. Wear gloves when casting the gel, because acrylamide can be absorbed through skin and is very carcinogenic.
12. Sometimes, despite of much devotion to assemble the casting stand, gels are leaking before polymerization. To prevent this, pipette 300 µl of a sealing gel (1 ml separating gel + 5 µl TEMED and 12.5 µl APS), wait 1–2 min for polymerization and cast the separating gel afterwards.
13. Gels should not be stored longer than 2 weeks, because this results in structurally instable gels.

14. Handle the PVDF membrane always with gloves, since proteins from your skin can attach to the surface and cause high background.
15. Sodium azide prevents microbial contamination, thus the primary antibody solution could be repeatedly used up to months when stored at –20°C. Do not add sodium azide to the secondary antibody, since this will inhibit the antibody-coupled horseradish peroxidase (HRP).
16. Stripping of membranes degrades antibodies as well as proteins of interest on the membrane. For that reason membranes should be stripped as less as possible. Try to alternate the species of secondary antibodies you use to avoid artifacts and background clouding of the band for the acetylated protein.

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Manuskript 4: Regulation of STAT3 and STAT1 by acetylation-phosphorylation cassettes

Regulation of STAT3 and STAT1 by acetylation-phosphorylationcassettes

Torsten Ginter[1], Christian Kosan[1],Carolin Bier[2], Roland H. Stauber[2], Andreas Henke[3], Thorsten Heinzel[1], and Oliver H. Krämer[1,*]

[1] Center for Molecular Biomedicine (CMB), Dept. of Biochemistry, University of Jena, Germany

[2] Dept. of Molecular and Cellular Oncology, University Hospital, Mainz, Germany

[3] Dept. of Virology and Antiviral Therapy, University Hospital, Jena, Germany

[*] Corresponding author: Oliver.Kraemer@uni-jena.de

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Abstract

Lysine acetylation controls interferon-dependent signaling by triggering STAT1 dephosphorylation through the phosphatase TCP45. Interestingly, STAT3 can be activated by acetylation. Acetylation-dependent inactivation of STAT1 can be mimicked by introducing additional glutamine residues (Q410/Q413) in its DNA binding domain (DBD). It is unclear whether the induction of such sites into STAT3 antagonizes its activation. Here, we show that introduction of an acetylation-mimicking arginine-glutamine-exchange at R414 of STAT3 ($STAT3^{R414Q}$) impairs tyrosine phosphorylation, nuclear translocation and STAT3/STAT3- or STAT1/STAT3-dependent target gene induction. RNAi against TCP45 shows that this loss of STAT3 functions depends on TCP45. Moreover, arginine-lysine-exchanges ($STAT3^{R414,417K}$; corresponding to STAT1 K410 and K413) attenuate the functions of STAT3. To further characterize the role of the DBD in STAT signaling, we analyzed STAT1 molecules with mutations in the N-terminal domain (NTD, $STAT1^{F77A,L78A}$). These cannot build π-π-stacks and hydrophobic interactions allowing to form homodimers and oligomers. While these STAT1 monomers resist TCP45-dependent dephosphorylation, lysine-to-glutamine mutations in their DBDs ($STAT1^{F77A,L78A*K410,413Q}$) prevent STAT1 signaling. These results suggest a molecularly transferable STAT phosphorylation-acetylation switch.

Introduction

Signal transducer and activator of transcription (STAT) proteins can be phosphorylated on tyrosine and serine residues by Janus and MAP kinases [1, 2]. While unphosphorylated STATs influence basal STAT-dependent gene expression, phosphorylated STAT homo-/heterodimers potently induce a specific set of genes[2-5]. STAT molecules consist of an N-terminal domain (NTD), a coiled coil, the DBD, a linker, an SH2 domain binding phosphorylated tyrosine residues, and the C-terminal domain (CTD).

Cytokines of the interferon (IFN)family fall into three classes [1, 2].IFN responses have to be regulated precisely to ensure homeostasis.When type I IFNs (IFNα/IFNβ)bind to their receptors, aSTAT1-STAT2-IRF9 complex is formed,translocates to the nucleus and activatesgene expressionemanating from IFN-stimulated response elements(ISRE). These promoters often control genes for

first line anti-viral defense, which positions STAT1 and STAT2 at the center of innate cellular immunity [1, 2, 6]. Type I IFNs also induce phosphorylation of further STATs and these can affect each other [7-9]. Type I and III IFNs evoke similar biological effects [10]. Binding of IFNγ to its receptor specifically induces STAT1 phosphorylation at tyrosine-701 (Y701; abbreviated as p-STAT1) to promote transcription from IFNγ-activated sites (GAS,typically nonameric palindromes of the sequence TTCNNNNAA)and immune modulation [1, 2, 6]. STAT3 is activated by cytokines such as IL6 or OSM,and also by IFNα.STAT3 is indispensable for embryonic development and STAT3 is often seen as a driver of oncogenesis [3].

Transcriptionally active STATs form tightly associated dimers via phospho-tyrosine–SH2 domain interactions and STATs also form oligomers. However, already in resting cells nearly all STAT1 in a cell exists as a dimer with STAT1 or other STATs, and this also holds true for STAT3 [3].Structural reorientation of the STAT1 dimer, generating a parallel to antiparallel conformational transition, permits STAT1 dephosphorylation by the T cell protein-tyrosine phosphatase (TCP45). Accordingly, STAT1 NTD mutants disrupting homodimer formation remain phosphorylated far longer than wild-type STAT1 [11].

Recent data demonstrate that STAT1 acetylation results in a switch to inactivate STAT1. IFNs, lipopolysaccharide, alloantigenic activation, and the chemotherapeutic cisplatin induce STAT1-dependent signaling sensitive to STAT1 acetylation. Accordingly, innate immunity and inflammatory processes are blocked by conditions evoking STAT1 acetylation in transformed and primary cells [9, 12-23].Pharmacological inhibition of histone deacetylases (HDACs) with specific histone deacetylase inhibitors (HDACi) blocks lysine deacetylationandincreases the acetylation of histones and non-histone proteins. Accordingly, these agents inhibit STAT1 signalingand this mechanism involves TCP45 [4].

Remarkably, STAT3 and STAT1 are both substrates for TCP45 [24], but STAT3 is not inactivated by acetylation [25, 26]. Acetylation of the N-terminal lysine residues K49, K87 or the C-terminal K685 rather enhances STAT3's transcriptional activity [25-27].STAT1 and STAT3 derived from a common primordial STAT and acetylation may have evolved as a regulator discriminating between these molecules. While they share 50% amino acid homology, differences are found within the DBDs of STAT1 and STAT3 [4]. A STAT1 mutant carrying lysine (K) to glutamine (Q) exchanges at positions 410/413 mimics the acetylated STAT1 ($STAT1^{K410Q,K413Q}$; abbreviated as

STAT1^{QQ}). TCP45 rapidly dephosphorylates this molecule, suggesting a critical role of these sites for STAT1 acetylation and dephosphorylation [9, 12, 13]. Interestingly, STAT3 cannot be acetylated at sites corresponding to STAT1 K410/K413, as these are arginines (R414 and R417) in STAT3 [13]. We hypothesized that mutation of such sites to glutamines (which imitate acetylation) or to lysines and treatment with HDACi should inactivate STAT3[4]. However, no formal proof for this idea has been provided so far. The opposite experiment, replacement of K410/K413 by arginine residues created a hyperactive STAT1 [13].

Weexpressed and analyzed mutant STAT1/STAT3 molecules in genetically defined cellular models lacking STAT1 (U3A cells) or STAT3 (PC3 cells). We found that mimicking acetylation of STAT3 at R414 by an R→Q amino acid exchange impairs STAT3-dependent signaling. We further demonstrate that the capability of STAT1 to form dimers together with this “cassette” negatively control STAT1.

Results

An acetylation cassette reduces IL6-induced STAT3 signaling

Although STAT1 is inactivated by acetylation, STAT3 has been found to be induced by acetylation [4]. Despite the extensive homology of STAT1 and STAT3, these STATs differ in their DBDs at sites found to affect acetylation of STAT1. In contrast to STAT1, STAT3 lacks lysines at positions 410 and 413. Sequence alignment analyses show that non acetylatable arginines at 414 and 417 correspondin STAT3 to these STAT1lysines(**Figure 1A**). This is valid for different species, e.g. *Homo sapiens*, *Mus musculus*, *Bos taurus*, *Xenopus laevis*, *Danio rerio*[28].

To test whether site-specific acetylation elements (i.e. K410 or K413 to Q exchanges within the STAT1 DBD) exist as transferable entities between STAT1 and STAT3, we changed the corresponding residue R414 in STAT3 to Q414 (R→Q mutation, from non-acetylatable to pseudo-acetylated, STAT3^{R414Q})(**Figure 1A**).STAT3 null PC3 cells are optimally suited to analyze STAT3-specific effects. To assess the biological functions of this moiety at more physiological expression levels, we stably reconstituted STAT3 null PC3 cells with STAT3^{R414Q}or wild-type STAT3. Notably, in response to IL6, wild-type STAT3 was far more pronouncedlyphosphorylated at tyrosine-705 (Y705) than STAT3^{R414Q}. Since acetylation marks STAT1 for TCP45-binding [9, 13], we asked whether phosphatase (PTP) inhibition could activate

$STAT3^{R414Q}$. The broad range PTP antagonist vanadate partially restored phosphorylation of $STAT3^{R414Q}$ in PC3 cells exposed to IL6 (**Figure 1B**).

Consistent with the phosphorylation results, $STAT3^{R414Q}$ was unable to accumulate in the nucleus after stimulation with IL6. Like acetylated STAT1 and $STAT1^{QQ}$, STAT3 is a substrate for TCP45 [24]. Therefore, we hypothesized that the R414Q mutation evoked TCP45-dependent latency of STAT3. Indeed, application of vanadate as well as a knockdown of TCP45 restored nuclear translocation of $STAT3^{R414Q}$ (**Figure 1C**). Likewise, shRNA-mediated attenuation of TCP45 allows phosphorylation of $STAT3^{R414Q}$ (data not shown).

Interestingly, $STAT3^{R414Q}$ shows no DNA binding of a GAS oligo as well, except for vanadate pretreated cells (**Figure 1D**). These findings suggest that, like $STAT1^{QQ}$ [13], $STAT3^{R414Q}$ has a cellular localization and structure comparable to wild-type STAT1, so that it can interact with receptors and JAKs. However, phosphorylating these molecules requires inhibition of TCP45.

IL6 induces phosphorylated STAT3 homodimers and STAT3-dependent transcription. When we analyzed transcriptional activation of the IL6-inducible STAT3-dependent reporter SIE-Luc in PC3 cells, we found that $STAT3^{R414Q}$ could not drive transcription from SIE-Luc (**Figure 1E**). These data suggest that the presence of a specific glutamine moiety in the STAT3 DBD (Q414) affect STAT3-dependent signaling.

The introduction of specific lysine in the STAT3 DBD attenuates STAT3-dependent signaling

We additionally generated STAT3 carrying the mutations R414→K/R417→K ($STAT3^{KK}$). $STAT3^{KK}$ mimics a particular feature of the STAT1 DBD – the lysine residues associated with acetylation-dependent STAT1 inactivation (**Figure 1A and 2A**) [9, 12, 13]. We asked how $STAT3^{KK}$, would affect SIE-Luc reporter activity. Remarkably, $STAT3^{KK}$is unable to induce IL6-dependent luciferase transcription (**Figure 2B**).

Both mutants, $STAT3^{R414Q}$ and $STAT3^{KK}$,indicate that a mimicked acetylation or a potential acetylation negatively regulate STAT3-dependent signaling. This is reminiscent for corresponding STAT1 sites (K410, K413), which undergo acetylation causing inactivity [4].

Quantitative effect of STAT3 on IFNγ-activated transcription elements

Phosphorylated STAT1 homodimers are formed in response to IFNγ to drive STAT1 homodimer-dependent transcription emanating from GAS sites. Nonetheless, STAT1 and STAT3 can be recovered from GAS sites when STAT3 is activated (e.g. by IL6 or IFNα/β) [7-9]. These observations prompted us to test a putative role for STAT3 on GAS-dependent transcription. Remarkably, we found that increasing the levels of STAT3 allowed IFNα-induced transcription of the GAS-Luc reporter in STAT1 null U3A cells (**Figure 2C**). This quantitative effect was specific as it was also evoked by IL6 which induces only p-STAT3 and not p-STAT1 in U3A cells [9] and because it was not generated by $STAT3^{R414Q}$ lacking an intact DBD (**Figure 2C**). Apparently, the quantity of STAT3 affects GAS-dependent transcription.

Consistent with the data we collected with the reporter SIE-Luc, we found that $STAT3^{R414Q}$ could not drive transcription from GAS-Luc and $STAT3^{R414Q}$fails to bind GAS DNA (**Figure 2C and 1D**). The inactivity of this STAT3 variant can hence be detected with different reporter systems.

Double lysine mutant $STAT3^{KK}$is affected by HDACi and shows reduced phosphorylation

When we co-transfected U3A cells with $STAT3^{KK}$ and the GAS-Luc reporter, we found that in comparison to STAT3 lacking lysine residues at these sites, $STAT3^{KK}$ is a weaker inducer of GAS-dependent transcription (**Figure 2D**). Since both, arginine and lysine are positively charged amino acids, we can exclude that the effects seen are due to a loss of positively charged side chains and an import defect *per se*. because $STAT1^{QQ}$ is impaired in cytokine-dependent signaling and since K→Q exchanges mimic acetylation, we addressed how the pan-HDACi trichostatin A (TSA) affects STAT3-dependent induction of the GAS-Luc reporter. We could confirm that STAT3 is acetylated upon treatment of cells with HDACi (data not shown). Also consistent with the literature [25, 26, 29]wild-type STAT3 became activated upon TSA plus cytokine co-treatment (**Figure 2D**).

Intriguingly, the IFNα-induced activity of $STAT3^{KK}$ was not induced upon TSA treatment (**Figure 2D**). Thus, introduction of particular lysine moieties at STAT3 K414 and K417, which unlike arginines that are present in wild-type STAT3, conferinhibitory effects of HDACi on STAT3. To further illuminate effects of such mutations, we analyzed phosphorylation and dephosphorylation kinetics of $STAT3^{KK}$

in comparison to wild-type STAT3. Upon immunoprecipitation of overexpressed STAT3, we observed less p-STAT3 and an accelerated dephosphorylation of $STAT3^{KK}$(**Figure 2E**). This finding might explain the poor induction of SIE- and GAS-luciferase reporters by $STAT3^{KK}$.

UBCH8 is regulated STAT3-dependently

The ubiquitin E3 ligase UBCH8 is induced by type I and type IIIFNs, and this process is mediated by ISRE and GAS promoter binding elements. GAS elementscan be bound by STAT3 as well (**Figure 1D and 2C-E**). We wondered if an alteration of STAT3 levels would influence UBCH8 expression. To test this, we used RNA interference against STAT3 in STAT1 reconstitutedU3A cells and could reveal that STAT3 is required for the induction of UBCH8 in IFNα-treated cells (**Figure 3A**).

Next, we analyzed whether an overexpression of $STAT3^{R414Q}$ in these cells might promoteUBCH8 protein levels. Remarkably $STAT3^{R414Q}$does not lead to an UBCH8 induction, even when $STAT3^{R414Q}$ is expressed at much higher levels than wild-type STAT3 (**Figure 3B**). Consistent with previous luciferase assay data $STAT3^{R414Q}$ is unable to induce the STAT3 target gene PIM1 (**Figure 3B**).

To completely exclude effects of endogenous STAT3, we established stable PC3 cell lines. In these cells we examined UBCH8 protein levels and could confirm inactivity of $STAT3^{R414Q}$regarding the IFNα-dependentinduction of UBCH8 (**Figure 3C**). These data suggest that STAT3 contributes to UBCH8 protein expression and that a STAT3 mutant, carrying a STAT1 acetylation module, loses this ability.

The acetylation cassette can be functionally transferred to an N-terminal STAT dimerization mutant

Next, we addressed whether dimerization of STAT1 is necessary for the inhibitory effect of the “acetylation cassette” Q410/Q413 in STAT1.A STAT1 molecule unable to dimerize with other STAT1 molecules couldallow addressing this issue (**Figure4A**). While STAT1 occurs as dimer already in resting cells and tissues [3], the NTD mutant $STAT1^{AA}$ exists as monomer and, in contrast to wild-type STAT1, resists dephosphorylation by TCP45[11]. We could confirm that $STAT1^{AA}$ remains phosphorylated more than four hours in cells stimulated with IFNγ or IFNα (**Figure4B**). We analyzed whether the pseudo-acetylation cassette K410Q/K413Q affected this regulation, i.e. whether $STAT1^{AA/QQ}$ does not have the prolongedtyrosine

phosphorylation ofthe NTD mutant STAT1^{AA}. When we compared the phosphorylation of STAT1$^{AA/QQ}$ and of STAT1^{AA}, we noted that compared to STAT1^{AA} the pseudo-acetylated mutant was far less phosphorylated at Y701 after treatment with IFNα or IFNγ (**Figure 4B**). The tyrosine PTP inhibitor vanadate significantly enhanced phosphorylation of STAT1$^{AA/QQ}$inIFN-induced cells. Apparently, theSTAT1 acetylation cassette (K410/413->Q) can be imposed as a module on STAT1^{AA}lacking an intact NTD.

Next, we analyzed transcriptional activation by STAT1 NTD mutants. We found that STAT1^{AA} significantly increased GAS-dependent reporter activation in IFNγ-treated U3A cells (**Figure 4C**). In agreement with its delayed dephosphorylation (**Figure 4B**), STAT1^{AA} wasa more potent transcription factor than wild-type STAT1 (**Figure 4C**). Sustained phosphorylation together with the failure to detect acetylation of STAT1^{AA} in response to IFNs (data not shown) agrees with acetylation of STAT1 counteracting its phosphorylation.Functional assessment of STAT1$^{AA/QQ}$showed that this STAT1 mutant could not drive the GAS-dependent reporter (**Figure 4D**). As anticipated from these data, expression of the IFN-inducible endogenous STAT1 target gene UBCH8was activated by STAT1^{AA}but not by p-STAT1$^{AA/QQ}$ in IFNγ-treated U3A cells (**Figure 4E**).

Concordant with its lack of ability to induce GAS-dependent transcription, STAT1$^{AA/QQ}$even failed to bind GAS DNA even when it was phosphorylated in vanadate plus IFNα or IFNγ treated U3A cells (**Figure 4F**). Recently, we reported that STAT1^{QQ} binds to DNA in a piggy-back complex with STAT3 [9]. Figure 4F argues that this mechanism does not exist for STAT1$^{AA/QQ}$. Two independent controls were done for this experiment.We checked for whether IFN plus vanadateinduced p-STAT1^{AA} and p-STAT1$^{AA/QQ}$equally, and whether IFNα/vanadate-activated p-STAT3 and if this could be recovered from the GAS oligonucleotide. Both molecules bound this response element independent of an intact STAT1 (**Figure 4F**). These data suggest that the underlying mechanism for the inactivity of STAT1$^{AA/QQ}$ relies on poor tyrosine phosphorylation and a lack of DNA binding to a GAS oligonucleotide. In contrast to our previous data for STAT1^{QQ}[9], the failure of STAT1$^{AA/QQ}$ to bind GAS DNA cannot be amended by STAT3. This is likely because p-STAT1^{QQ} binds to DNA with STAT3 [9], but no interaction between STAT1$^{AA/QQ}$ and STAT3 is formed on GAS DNA.

Relevance of the STAT1 NTD for interactions with STAT2 and anti-viral protection

Activated STAT1/STAT2 dimers confer innate immunity via activation of ISRE-dependent transcription. Although pre-formed STAT1/STAT2 complexes exist irrespective of IFN stimulation [3], we could exclude residual binding of STAT1 NTD mutants to STAT2 (**Figure 5A**). Hence, we conclude that the F77A/L78A mutations in $STAT1^{AA}$ change the STAT1 molecule in a way preventing its interaction with STATs1,-2,-3, i.e. that the NTD is not only necessary for homodimerization but equally for heterodimerization.

Most anti-viral genes are induced by a STAT1-STAT2 heterodimer [1, 2, 6].Vesicular stomatitis virus (VSV) is a complex stimulus that is highly sensitive to IFNα and routinely used to assay IFN-dependent cellular antiviral activities [9, 15]. Compared to $STAT1^{AA}$, $STAT1^{AA/QQ}$ less efficiently protected cells from this agent (**Figure 5B**). These data agree with our data showing higher phosphorylation and basal transcriptional activity of $STAT1^{AA}$ (**Figure 4B**-**D**). The cytoprotective effect of $STAT1^{AA}$ was though not augmented by IFNα or IFNγ, which agrees with its inability to bind STAT2 (**Figure 5A**). Thus, formation of dimers and site-specific modifications within the STAT1 DBD mediate cellular reactions during VSV infection.

Discussion

Our findings on STAT1 and STAT3demonstrate that K→Q mutations within their DBDs accelerate dephosphorylation and blocktheir nuclear import and DNA binding. These data are reminiscent of the effects HDACi exert on STAT1 asacetylated STAT1 largely resists cytokine-induced phosphorylation [4]. STAT1 K410 and K413 arelocated in the surface-exposed DBD. Modifications at this site may increase the off-rate of STAT1from DNA and the dephosphorylation of pY701 by TCP45[11, 13, 30].

Our data suggest that “motifs”, which can become acetylated or which mimic acetylated residues, control STAT1 and STAT3. **Figure 5C**depicts the differences between STAT1 versus STAT3 and it emphasizesa role for lysine to glutamine residues mimicking acetylation. Remarkably, STAT2 and STAT3 do not contain lysine residues at sites corresponding to STAT1 K410/K413 [4], and STAT2 and STAT3can be positively controlled by acetylation[9, 15, 25, 26]. It is surprising that

single amino acid exchanges within their DBDs alter the regulation of STAT1 and STAT3. However, such results are not unprecedented. Acetylation cassettes that differentially affect gene expression are reported for p53 [31]. It appears that neomorphic features of STAT1, which like the other STATs arose from gene duplications that happened before the divergence of insects and vertebrates[32], were endowed with acetylation-dependent control sites. Our data show that "acetylation cassettes" containing glutamine or lysine residues at positions 410/413 (in the STAT1 DBD) are transferable. They mediate phosphatase-dependent inactivation of STAT3 which is otherwise resistant to acetylation-dependent inactivation and can even be further activated by acetylation of lysine residues [25, 26]. Such site-specific mutations also render STAT3 sensitive towardsHDACi.

Likewise, the STAT1 acetylation cassette represented in the DBD of $STAT1^{QQ}$ appears transferable to STAT1 which cannot dimerize ($STAT1^{AA}$) and resists TCP45-dependent dephosphorylation. Thus, introduction of K410→Q/K413→Q mutations into $STAT1^{AA}$ yielded $STAT1^{AA/QQ}$, a molecule combining STAT1 pseudo-acetylation and its inability to bind other STAT1 molecules (**Figure 5C**).

STAT1 K410 and K413 belong to the STAT1 NLS/NES. STAT1 molecules carrying K410A/K413A exchanges are phosphorylated but import-deficient [33]. These data do not disagree with our conclusions and models [9, 13]. Replacing lysine residues by alanine moieties ties in with a lost positive side chain required for nuclear import and at the same time prevents putative acetylation of the lysine side chain. Since forced phosphorylation of $STAT1^{QQ}$ permits nuclear entry [9, 13], other sites should also dictate interaction of STAT1 and importins. Indeed, STAT1 with L407 or L407+L409 mutated, but not STAT1 with charge-modifying K410/K413 mutations, displays strict cytosolic localization [30, 34, 35]. Importin α binds the NLS of phosphorylated STAT1 and STAT3 molecules and translocates them to the nucleus with importin β. The STAT1 residues L407, K410, and K413 appear all necessary to interact with importin α5 (NPI-1) and with importin α7 [33, 34, 36].

Others found that R414 and R417 are important for the cytokine-induced nuclear import of STAT3. Deletion or changing these side chains to alanine residues (i.e. an amino acid with a very short side chain) does not cause constitutive dephosphorylation of STAT3 [36, 37].Nuclear import of such mutants is though not possible. We found that mutant STAT3 with lysine-to-glutamine exchanges in its DBD cannot accumulate in the nucleus of cells exposed to IL6. The fact that application of

this cytokine plus vanadate or attenuation of TCP45 allows nuclear transit of $STAT3^{R414Q}$ in a STAT3 null background argues against a general structural defect of this protein. These observations rather suggest that STAT molecules carrying pseudo-acetylated modules are principally intact and permissive for phosphorylation but blocked via rapid dephosphorylation catalyzed by TCP45. Interestingly, similar to $STAT1^{QQ}$ mutant [9, 13], a STAT1 mutant unable to enter the nucleus and to bind DNA (termed STAT1 DNA ^{minus}, V426D/T427D exchanges) can also be forced to enter the nucleus upon co-stimulation with IFNγ and vanadate. Moreover, a STAT1 K410/K413→E/E mutant also fails to accumulate in the nucleus upon IFNγ stimulation[30].

As for STAT1, where L407 is relevant in addition to K410/K413 [33, 34, 36], R214/R215 are STAT3 sites mediating contact with importins α5/α7. However, STAT3 R414/R417 seem not to directly bind these importins, but maintain an import-competent structure of STAT3 [36, 37]. Of note, other reports show that importin α3 and importin β1/Ran-GTPase mediate a high, phosphorylation-independent nuclear import of STAT3 [38, 39]. Furthermore, one cannot exclude that acetylation or mimicking this posttranslational modification of one site affects posttranslational modifications other than phosphorylation (even another acetylation event). Detailed analyses of STAT1 and STAT3 within the chromatin context may also be necessary to exactly define the role of certain DNA/protein complexes for STAT1 dephosphorylation by TCP45 and perhaps other PTPs.Such complex interactions and dynamic control mechanisms may well explain why mass spectrometry analyses have found an increasing number of previously overlooked STAT3 acetylation sites over the last years [27, 28].

The mutations we introduce into STAT3 do not occur *in vivo*. However, the main focus of the work presented is to find out whether STAT1 “acetylation cassettes”are transferable as modular units. This analysis also reveals if particular differences in the DBDs of STAT1 and STAT3 are relevant for their signaling. We demonstrate that indeed the presence of one acetylation-mimicking glutamine residue within the STAT3 DBD causes similar effects as the corresponding mutation in STAT1, namely the inability to respond appropriately to cytokine stimulation. Furthermore, the STAT1 lysine moieties which can mimic acetylation upon mutation towards glutamine (K410→Q/K413→Q) were identified as acetylated sites in a mass spectrometry approach [28]. Moreover, all experiments were done in STAT1 or STAT3 null

backgrounds which allow conclusions about the mutants independent of any endogenous background issues. The new cell lines reconstituted with STAT1 and STAT3 variants we introduce here will be useful for additional studies. Analyzing these cells we noted that expression of UBCH8 in IFNα-treated cells is controlled by both, STAT1 and STAT3. While a lot of IFN-regulated genes are known, UBCH8 is still one of the few examples which are induced by IFNα and STAT3[8, 40].

Our data further propose that the F77A/L78A mutations in $STAT1^{AA}$prevent its interactions with STATs1,-2,-3. These data reveal that an intact NTD is necessary for homodimerization and oligomerization [11], and equally for heterodimer formation. The π-π-electron stacks of two opposing phenylalanines and the leucine side chains may form hydrophobic interaction surfaces relevant for these interactions. Curiously, STAT3 molecules also oligomerizedependent on their N-termini (residues 1-135) [38]. Further work is required to decipher which amino acid side chains in STAT2 and STAT3 associate with STAT1.

STAT1 and STAT3 show evolutionarily conserved differences in their DBDs and our datapropel the concept that distinct posttranslational lysine acetylation of STAT1 versus STAT3 emerged during evolution as a divergent control mechanism. Interestingly, STAT orthologues from the slime mold *Dictyostelium discoideum* and the nematode *Caenorhabditis elegans* lack an amino-terminal oligomerization domain. This suggests that the primordial STAT lacked this domain and that it became accreted later in evolution to provide additional regulation within the context of cytokine responses[32]. Analyzing the *Caenorhabdites elegans* STAT orthologue STA-1 reveals that the DBD is not always positively involved in STAT activation [32]. While the DBD is a very necessary feature of JAK-STAT signaling in vertebrates, it can affect STAT tyrosine phosphorylation, dependent on the presence of lysine (STAT1) or arginine (STAT3) residues. Our data suggest that acetylation confers different functionalities to closely related transcription factors that evolved from a common primordial STAT ancestor.

Materials and methods

Cell Lines, transfections, microscopy

Cells were maintained, treated and transfected as described [12]. Whereas transfection of excessive amounts of STAT1 causes cell death and outcompetes

TCP45, stable expression of STAT1 in U3A cells was achieved comparable to its endogenous level in parental 2fTGH cells [13]. Immunofluorescence staining for STAT3-V5 was done as described [12]. Cells were incubated with 10^3 U/ml IFNα, 50 ng/ml IFNγ, 50 ng/ml IL6, or 0.1-1 μM trichostatin A (TSA). Vanadate was used as noted in [12, 13]. PC3 prostate carcinoma cells (STAT3-/-) were transfected with STAT3-V5 or mutants as stated in legends and selected with 900 μg/mL G418.

Plasmids, siRNA

Human STAT1α constructs were described before [12].

We mutated the N-terminal STAT1 dimerization surface (N-terminal domain, NTD) by replacing phenylalanine F77 and leucine L78 by alanines ($STAT1^{AA}$) (**Figure 4A**). STAT1α with F77A/L78A was obtained with 5-GCTTTTCTTTGGAGAATAACGCCGCGCTACAGCATAACATAAGG-3 and 5-CCTTATGTTATGCTGTAGCGCGGCGTTATTCTCCAAAGAAAAGCG-3. Human STAT3α cDNA was cloned into pcDNA3V5HIS TOPO and mutated STAT3 R414Q was created with 5-CACTTGACCCTGCAGGAGCAGAGATGTGGGAATGGGGGC-3 and 5-CATTCCCACATCTCTGCTCCTGCAGGGTCAAGTGTTTG-3. $STAT3^{KK}$ wascreatedwith 5-CTTGACCCTGAAGGAGCAGAAATGTGGGAATGGGGGC-3 and 5-CATTCCCACATTTCTGCTCCTTCAGGGTCAAGTGTTTG-3. For STAT3 knock down 5-AUUGUGCUGAUAGAGAACATT-3 siRNA was used.

Luciferase reporter assays

Luciferase reporter assays were performed as in [13]. Data shown are representative for independently repeated experiments.

Antibodies, drugs and chemicals

Antibodies were purchased from Santa Cruz Biotechnology (STAT1, sc-346/sc-417; p-STAT1, sc-7988-R; STAT2, sc-476; STAT3, sc-482; p-STAT3, sc-8059; HA, sc-7392/sc-805); Sigma (Tubulin, T5168); Roche (TCP45, CF4-1D); Covance (HA, 11-MMS-101P) and Abgent (UBCH8, AP2118b). Drugs and chemicals were from the sources listed in [12, 13]. IFNγ and IL6 were purchased from Immunotools.

Antiviral assay

The antiviral effect of IFNα and IFNγ was determined by measuring the cytopathic effect (cpe) of vesicular stomatitis virus (VSV) on stably transfected U3A cells. Cells wereincubated with IFNs for 24 h in 96-Well plates. Supernatant was removed and cells were infected with VSV at a multiplicity of infection (MOI) of 0.1 or 1. The choice of MOI depended on the tolerance of the stable cell linesused. Cells were incubated

with VSV for 24 h. Plates were washed carefully with PBS to remove cellular debris of necrotic cells. Remaining cells were fixed and stained with 0.2% crystal violet, 20% ethanol, and 3.5% formaldehyde in ddH_2O for 24 h. Stained cells were washed three times with water and dried. Remaining dye was extracted using lysis buffer (48% ethanol, 2% 1 N HCl, and 0.9 g sodium citrate in ddH_2O) for 20 min. Absorbance was measured on a Dynatech MR5000 plate reader at 550/630 nm. Each point of measurement was done as quadruplicate and normalized to uninfected control cells (set as 100%). Results represent arithmetical means of three independent assays.

Preparation of cell lysates, immunoprecipitation, immunoblotting, ABCD-assay (Avidin-Biotin-Coupled DNA-Assay)

These methods were described recently [12, 13].ABCD assay was performed with the GAS site containing oligonucleotides 5-GAGACTCAGTTTCCCGTAAATCGTCCAGTTTCCCGTAAAGACTATGC-3 and 5-GCATAGTCTTTACGGGAAACTGGACGATTTACGGGAAACTGAGTCTC-3 or irrelevant oligonucleotides.

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Figure Legends

Figure 1. Acetylation-like modules can be transfered between STAT1 and STAT3

(**A**) Alignment of *Homo sapiens* STAT1 and STAT3 protein sequences within their DBDs.Lysine residues causing inactivation of STAT1 upon exchange to acetylation

mimicking glutamines. STAT3 containsarginine residues at corresponding sites, which cannot become acetylated.

(**B**) PC3 cells stably expressing STAT3 wild-type (WT) and STAT3 ^{R414Q} (R414Q) were stimulated with IL6 (+) and vanadate (+). P-STAT3/STAT3 were detected by Western blot.

(**C**) Immunofluorescence microscopy revealed impaired nuclear translocation of STAT3^{R414Q} (R414Q). Stably transfected PC3 cells with STAT3 WT or R414Q were transfected with shRNA for TCP45 (shCtr, irrelevant shRNA). Cells were pretreated with 1 mM vanadate for 30 min and with IL6 for 180 min; scale bar 10 µm.

(**D**) ABCD assay reveals impaired GAS DNA binding of STAT3^{R414Q}. PC3 cells were transiently transfected with wild-type STAT3 (WT) and STAT3^{R414Q} (R414Q). Cells were pretreated with 1 mM vanadate for 30 min (+) and stimulated with IL6 (+) for 1 h. Protein levels of p-STAT3 and STAT3 were detected by Western blot. GRE, glucocorticoid-responsive element served as negative control. Input, 10 % of lysate

(**E**) STAT3^{R414Q} could not activate a SIE-Luc reporter. PC3 cells were transiently transfected with STAT3 wild-type (WT), STAT3^{R414Q} (R414Q)or empty vector pcDNA3.1 (3.1). Immunoblot ensured equal STAT3 levels.

Figure 2. Transcriptional activity of pseudo-acetylated STAT3

(**A**) Schematic view of used STAT3 argininemutants.

(**B**) SIE-Luc reporter assay reveals impaired transcriptional activity of STAT3^{R414,417K} (KK). PC3 cells were transiently transfected with STAT3 wild-type (WT), STAT3^{R414,417K}(KK) or empty vector pcDNA3.1 (3.1) and stimulated with IL6 for 8 h.

(**C**) GAS-Luc assay in U3A cells transiently transfected with STAT3, STAT3^{R414,417K} (KK), STAT3^{R414Q} (R414Q) or pcDNA3.1 (3.1). Stimulation with IL6 (+) and IFNα (+) was done for 24 h.

(**D**) U3A cells were transiently transfected with GAS-Luc reporter, STAT3 (WT), STAT3^{R414,417K} (KK) or pcDNA3.1 (3.1). Cells were stimulated 24 h with IFNα and 100 nM TSA.

(**E**) Different phosphorylation and dephosphorylation kinetic of STAT3 mutant. U3A cells were transiently transfected with STAT3 wild-type (WT) and STAT3^{R414,417K} (KK). Cells were stimulated for 30 min with IL6 (+), followed by a medium change. STAT3 was immunoprecipitated (IP) using V5-tag and subsequently analysis of p-

STAT3 and STAT3was done by immunoblot. Input, 5 % of lysate; Pre, pre-immune serum.

Figure 3. Impact of acetylation mimicking STAT3 on target genes

(**A**) STAT3 positively affects UBCH8 expression. U3A cells were transiently transfected with siRNA against STAT3 (siS3), control siRNA (siCtr), STAT1 wild-type and empty vector pcDNA3.1 (3.1). Cells were stimulated with IFNα (+) for 24 h. Protein levels of STAT1, STAT3 and UBCH8 were analyzed by Western blot. Tubulin served as loading control.

(**B**) Overexpression of $STAT3^{R414Q}$ demonstrates ineffective induction of target genes. U3A cells transiently transfected with wild-type STAT1, STAT3 (WT), $STAT3^{R414Q}$ (R414Q) and empty vector (3.1) were stimulated with IFNα for 24 h (+). UBCH8, PIM1, V5 and tubulin protein levels were determined by immunoblot.

(**C**) PC3 cells stably expressing GFP, wild-type STAT3 (WT) or $STAT3^{R414Q}$ (R414Q) indicate negative regulation of UBCH8 by $STAT3^{R414Q}$. Protein levels of STAT3, UBCH8 and tubulin were assessed by Western blot.

Figure 4. Functional inactivation of the acetylation mimicking STAT1 monomer

(**A**) Model illustrating STAT1 monomeric variants.

(**B**) Phosphorylation of $STAT1^{AA/QQ}$ (AA/QQ) could be forced with IFNα/γ (+) and preincubation with vanadate (30 min). U3A cells expressing STAT1 (AA or AA/QQ) were treated as indicated. P-STAT1/STAT1 levels were analyzed by immunoblot.

(**C**) Luciferase assay was done with transiently transfected U3A cells. Cells were transfected with GAS-Luc, STAT1(WT, wild-type), $STAT1^{AA}$(AA), pcDNA3.1(3.1). After stimulation of cells with IFNγ (24 h) luciferase activities were measured (reporter activation by wild-type STAT1 is set as 100%); statistical significance over controls $p<0.001$. Western blot verifies equal STAT1 levels.

(**D**) Same as in (**C**), but including $STAT1^{AA/QQ}$ (AA/QQ) and co-incubation with vanadate.

(**E**) U3A cells stably expressing $STAT1^{AA}$ (AA) and $STAT1^{AA/QQ}$ (AA/QQ) differentially affect UBCH8 expression. Cells were stimulated with IFNγ (+) for indicated periods. Protein levels of STAT1, UBCH8 and tubulin were determined by Western blot.

(**F**) ABCD assay revealed DNA binding of monomeric STAT1. Cells pretreated with vanadate (30 min) were stimulated with IFNα/γ (1 h). Immunoblots were probed as stated.Input, 10 % of lysate.

Figure 5. Lack of interaction with STAT2 does not impair the STAT1 monomer
(**A**) HA IPs from 293T cell lysates containing HA-STAT1 (WT), HA-STAT1^{AA}, or HA-STAT1$^{AA/QQ}$ were probed against STAT2 and HA; input, 10% of lysate. Cells were stimulated with IFNα/γfor 45 min.
(**B**) Antiviral assay assesses activity of STAT1^{AA} (AA) and STAT1$^{AA/QQ}$(AA/QQ). Stably transfected U3A cells were incubated with IFNα/γ for 24 h before infection with VSV (MOI 0.1).
(**C**) Summary of the characteristics of the acetylation cassette in different contexts. Leftpanel: STAT3 containing an arginine to glutamine (R414Q) mutation remains inactive, due to enhanced dephosphorylation by TCP45. Right panel: A monomeric STAT1-NTD mutant is overactive, due to hindered dephosphorylation. This status could be inverted byintroduction of glutamine residues (K410,413Q) in its DBD. Inhibition of TCP45 enables tyrosinephosphorylation of STAT3 and STAT1 glutamine mutants, mimicking acetylation.

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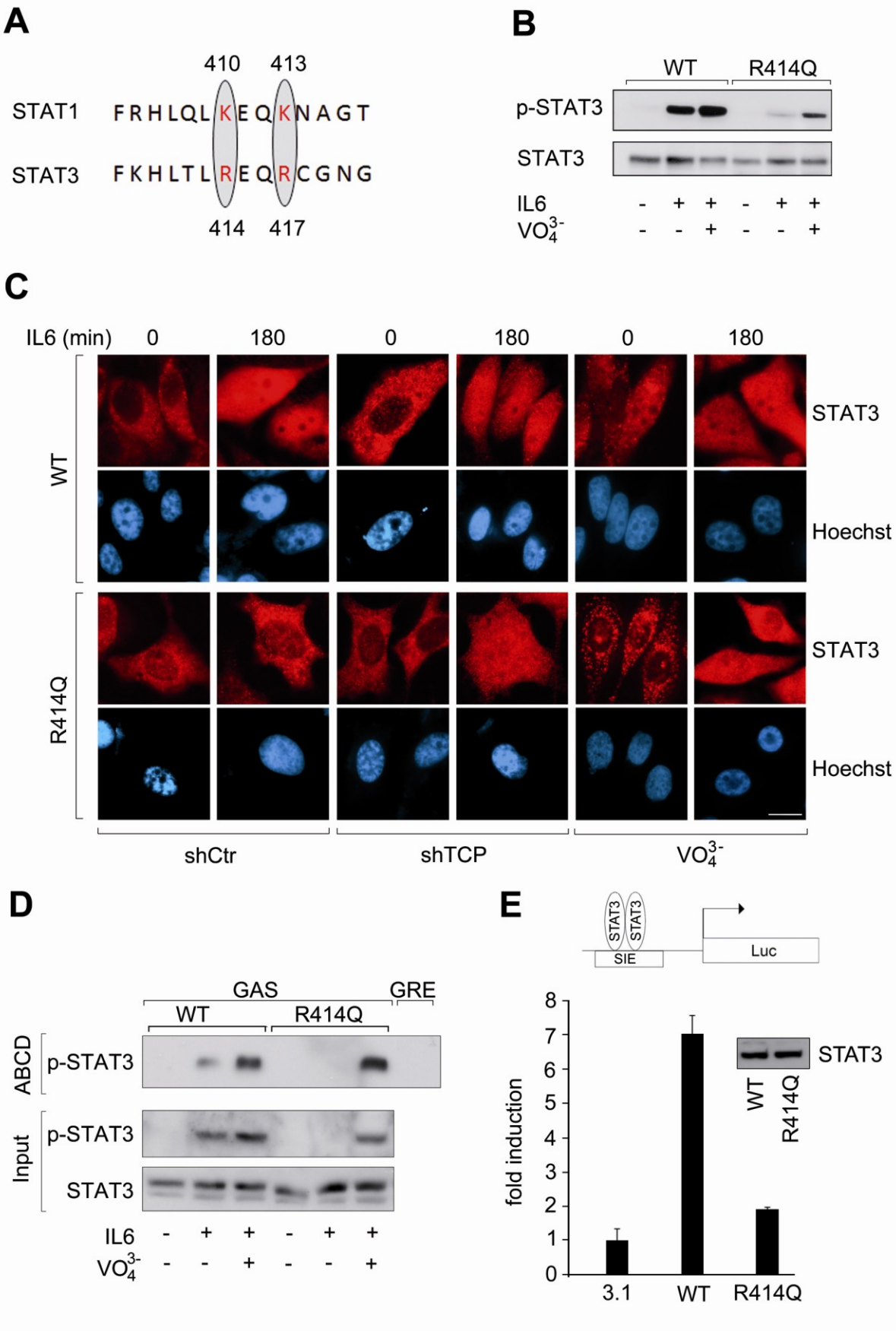
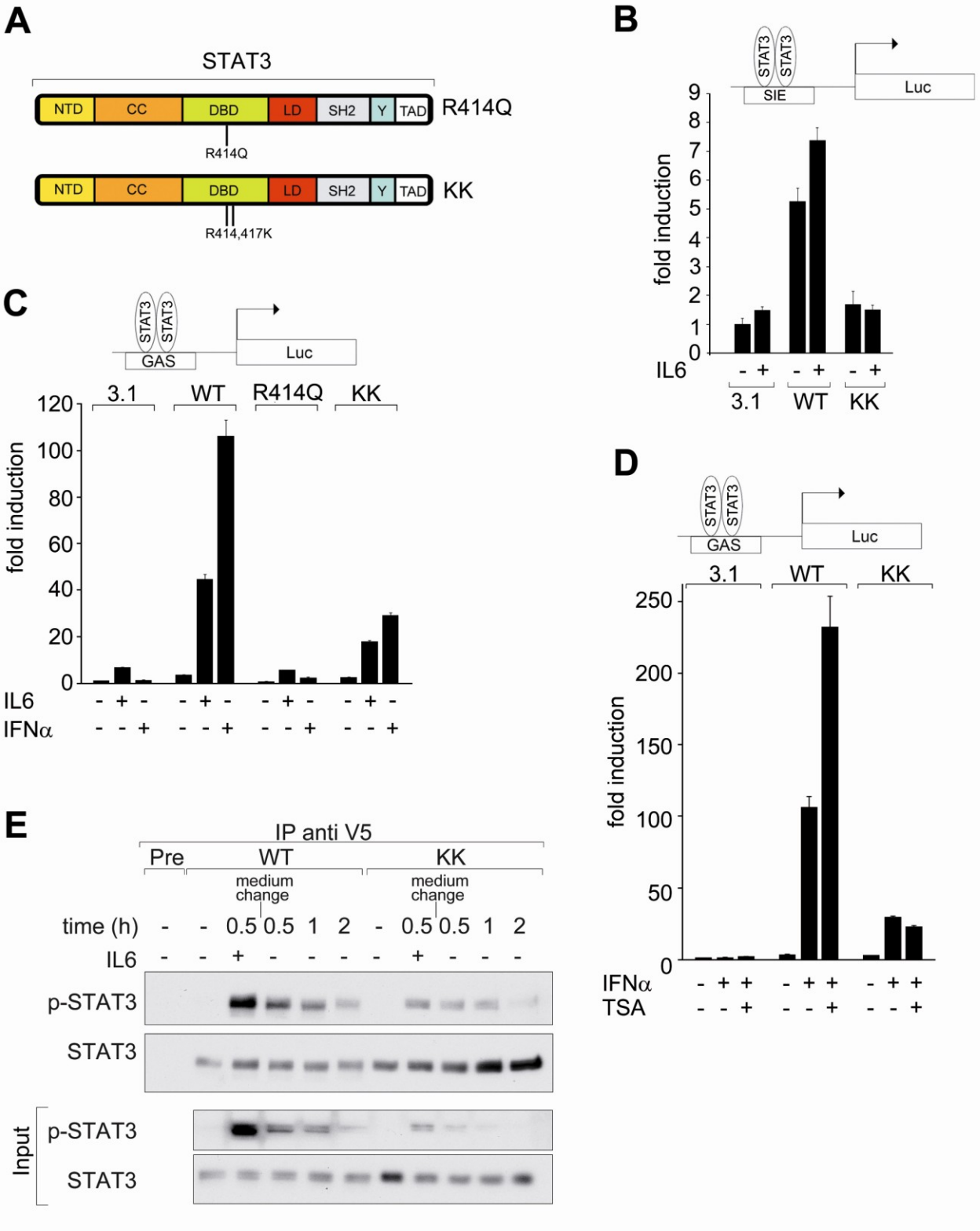
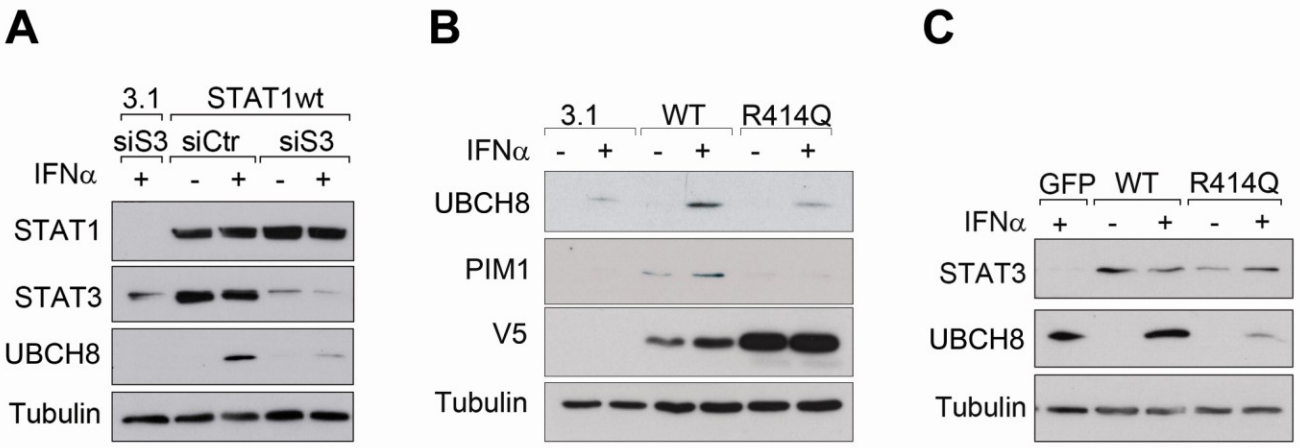
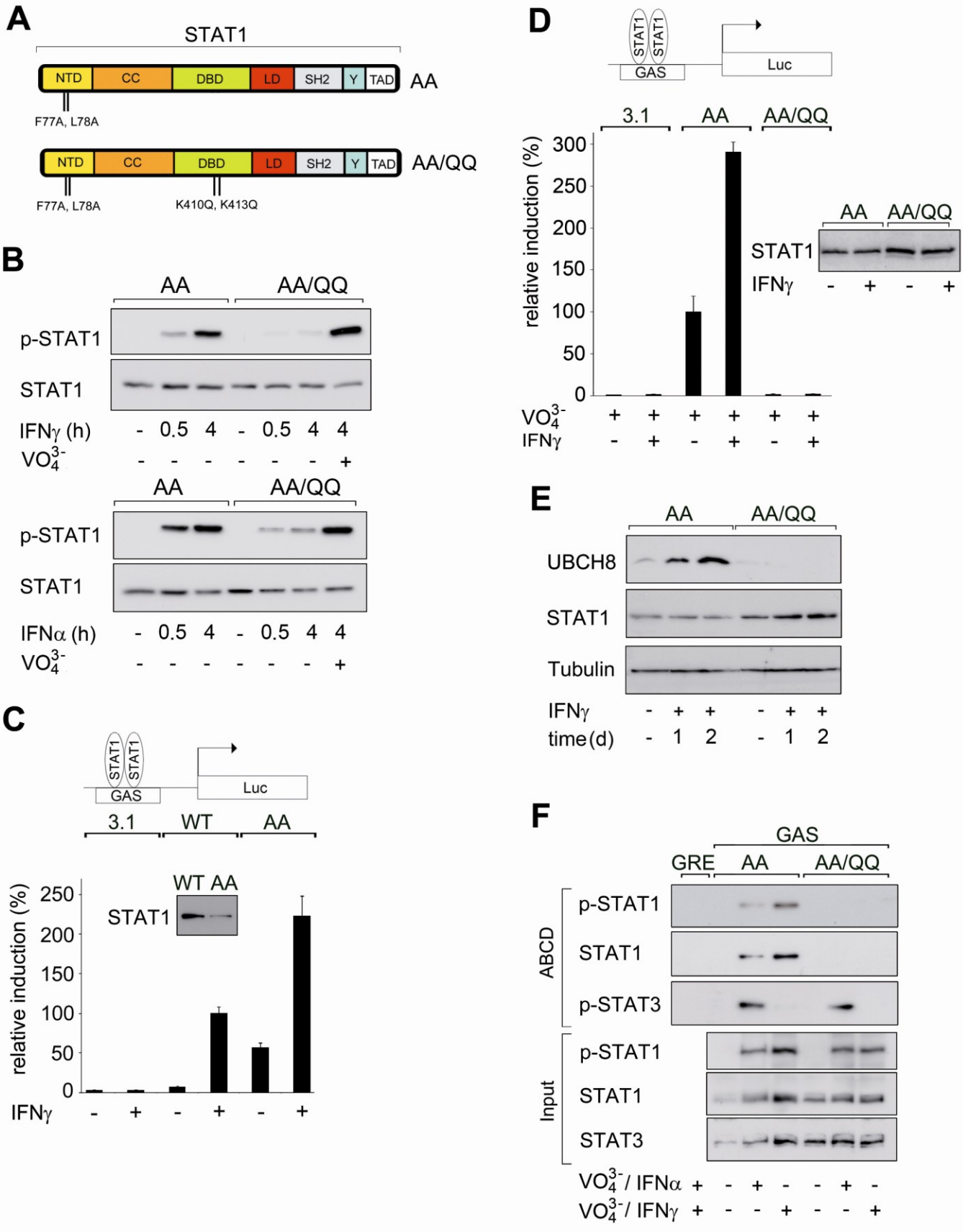
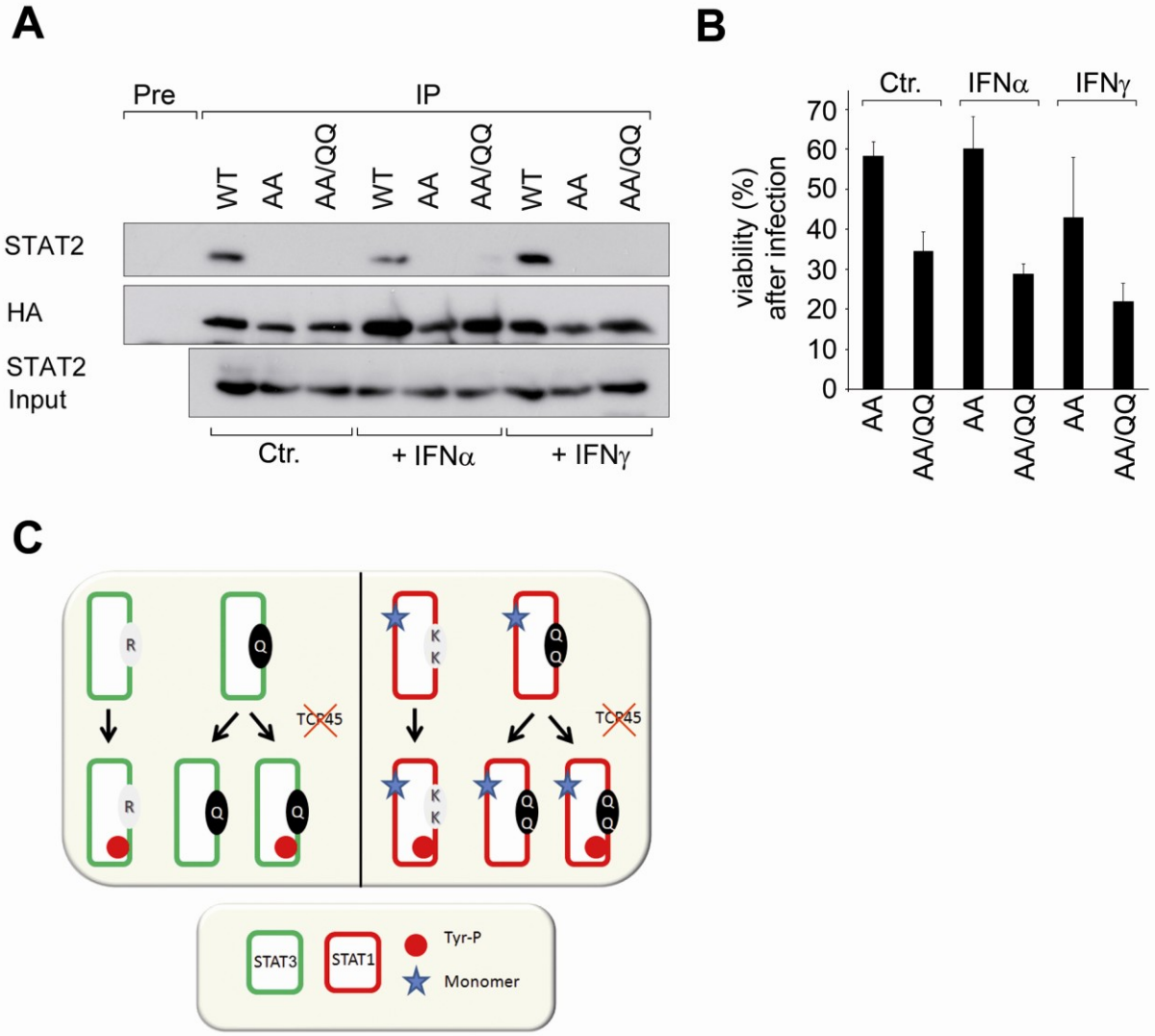

A
410
413
STAT1
FRHLQLKEQKNAGT
STAT3
FKHLTLREQRCGNG
414
417
B
WT
R414Q
p-STAT3
STAT3
IL6
VO4 3-
C
IL6 (min)
0
180
WT
R414Q
STAT3
Hoechst
shCtr
shTCP
VO4 3-
D
GAS
GRE
WT
R414Q
ABCD
p-STAT3
Input
STAT3
IL6
VO4 3-
E
STAT3
SIE
Luc
fold induction
3.1
WT
R414Q


A
STAT3
NTD
CC
DBD
LD
SH2
Y
TAD
R414Q
R414Q
KK
R414,417K
B
STAT3
STAT3
SIE
Luc
fold induction
IL6
3.1
WT
KK
C
GAS
Luc
3.1
WT
R414Q
KK
fold induction
IL6
IFNα
D
GAS
Luc
3.1
WT
KK
fold induction
IFNα
TSA
E
IP anti V5
Pre
WT
KK
medium change
time (h)
IL6
p-STAT3
STAT3
Input
p-STAT3
STAT3


A
3.1
STAT1wt
siS3
siCtr
siS3
IFNα
+ - + - +
STAT1
STAT3
UBCH8
Tubulin
B
3.1
WT
R414Q
IFNα
- + - + - +
UBCH8
PIM1
V5
Tubulin
C
GFP
WT
R414Q
IFNα
+ - + - +
STAT3
UBCH8
Tubulin


A
STAT1
NTD
CC
DBD
LD
SH2
Y
TAD
AA
F77A, L78A
AA/QQ
K410Q, K413Q
B
AA
AA/QQ
p-STAT1
STAT1
IFNγ (h)
- 0.5 4 - 0.5 4 4
VO4 3-
IFNα (h)
C
STAT1
STAT1
GAS
Luc
3.1
WT
AA
WT AA
relative induction (%)
250
200
150
100
50
0
IFNγ
D
3.1
AA
AA/QQ
relative induction (%)
300
250
200
150
100
50
0
VO4 3-
IFNγ
E
UBCH8
Tubulin
time (d)
F
GAS
GRE
ABCD
Input
STAT3
VO4 3-/ IFNα
VO4 3-/ IFNγ


A
Pre
IP
WT
AA
AA/QQ
WT
AA
AA/QQ
WT
AA
AA/QQ
STAT2
HA
STAT2
Input
Ctr.
+ IFNα
+ IFNγ
B
Ctr.
IFNα
IFNγ
viability (%)
after infection
70
60
50
40
30
20
10
0
AA
AA/QQ
AA
AA/QQ
AA
AA/QQ
C
R
Q
TCP45
K
K
Q
Q
STAT3
STAT1
Tyr-P
Monomer

5. Diskussion

In den letzten Jahren sind zunehmend Beispiele für die Acetylierung von Nicht-Histon-Proteinen entdeckt worden. Die Lysinacetylierung kann dabei entscheidende Proteinfunktionen, wie Lokalisation, DNA-Bindung und Proteinstabilität verändern. Bemerkenswert ist, dass nur ein sehr geringer Anteil eines Proteins acetyliert sein kann und dieser dennoch biologisch relevante Prozesse steuern kann. In Wieczorek et al. wird ein umfassender Überblick über die Acetylierung der STAT-Proteine gegeben [Wieczorek, 2012]. Weiterhin wurde ein Buchkapitel über den Nachweis dieser Modifikation verfasst [Ginter, 2013] und erstmals wurde eine IFNγ-induzierte Acetylierung von humanem STAT1 nachgewiesen, welche die Funktion von STAT1 hemmt [Ginter, 2012].

5.1 HDACi bewirken eine Dephosphorylierung von STAT1

Die Expression und Aktivität von HDACs ist in vielen Tumoren fehlreguliert, weshalb Inhibitoren für diese Enzyme in intensiver Entwicklung sind. HDACi sind somit vielversprechende Krebstherapeutika, aber auch zur Behandlung von unkontrollierten Immunreaktionen erwägenswert [Buchwald, 2009]. Viele Studien haben gezeigt, dass HDACi den STAT1/STAT2 vermittelten Signalweg unterdrücken, welcher für das adaptive und angeborene Immunsystem maßgeblich ist [Genin, 2003, Klampfer, 2003, Nusinzon, 2003, Chang, 2004]. STAT1 wird durch HDAC1,2,3 und 4 (Tabelle 1) deacetyliert und eine Hemmung durch die HDACi, Butyrat, Valproinsäure (VPA) Trichostatin A (TSA) und SAHA (Vorinostat) bewirkt eine verminderte STAT1 Phosphorylierung [Klampfer, 2004, Krämer, 2009]. Unsere Gruppe konnte erstmals zeigen, dass STAT1 ein direktes Ziel der Acetylierung ist und dass IFNα-induzierte Tyrosinphosphorylierung dadurch vermindert wird [Krämer, 2009].

In der vorliegenden Arbeit war es mir möglich eine durch HDACi hervorgerufene Reduzierung der IFNγ-induzierten STAT1-Phosphorylierung nachzuweisen [Ginter, 2012]. Dies gelang durch Verwendung von TSA, einem pan-HDACi und zwei Klasse I HDAC spezifischen Inhibitoren, Valproinsäure (VPA) und MS-275 (Etinostat) [Witt, 2009]. Die Beobachtung konnte in diversen Zelllinien (HEK 293T, NB4) und in primären murinen Knochenmarkzellen (BMC) sowie in davon abgeleiteten dendritischen Zellen (BMDC) verifiziert werden. Zusätzlich konnte der negative Effekt eines HDACi auf die STAT1-Phosphorylierung in primären humanen Zellen, mit Hilfe von Nabelschnurendothelzellen (HUVEC) nachgewiesen werden [Ginter, 2012]. Die genannten Daten legen nahe, dass die HDACi-vermittelte Reduktion der STAT1-Phosphorylierung ein allgemeingültiger Effekt ist. Da fehlreguliertes STAT1 bei chronischen Entzündungen und bei vielen

Autoimmunerkrankungen eine Rolle spielt [O'Shea, 2012, Takezaki, 2012], könnte eine Inaktivierung über HDACi von therapeutischem Interesse sein.

5.2 IFNγ-induziertes Acetyl-STAT1 interagiert verstärkt mit TCP45

Typ I und II IFNs induzieren die Phosphorylierung des Tyrosins 701 in STAT1. Diese Phosphorylierung ist von zentraler Bedeutung für die Aktivierung des STAT-Dimers (siehe 2.1.3). Die Inaktivierung der STAT1 Signalkaskade ist ebenfalls wichtig, um eine Überaktivierung von STAT1 zu vermeiden oder eine spätere, erneute Stimulation zu erlauben. Eine Möglichkeit der Inaktivierung bildet die Dephosphorylierung durch TCP45 (siehe 2.1.6) [ten Hoeve, 2002]. In vorausgegangenen Arbeiten konnten wir zeigen, dass die IFNα-induzierte STAT1-Phosphorylierung zu einer nachgeschalteten STAT1-Acetylierung führt [Krämer, 2009]. Diese Acetylierung vermittelt eine Latenzzeit, in der keine erneute Phosphorylierung stattfinden kann. In derselben Arbeit wurde mit TCP45 substrate trapping Mutanten eine verstärkte Bindung dieser Phosphatase an acetyliertes STAT1 beschrieben.

Der Nachweis der Acetylierung als PTM erfordert die Einhaltung verschiedener Bedingungen. Ein stringenter Lysepuffer und die Zugabe von HDACi zu Wasch- und Lysepuffer sind beispielsweise unverzichtbar. Zusätzlich sind bei IFN-induzierter Acetylierung lange Stimulationszeiten nötig [Ginter, 2013]. Außer Acht lassen dieser Vorkehrungen kann zu falsch-negativen Ergebnissen und Missverständnissen in der Forschergemeinschaft führen, wie die Arbeit von Antunes et al. zeigt [Antunes, 2011].

In mit IFNγ stimulierten HEK 293T Zellen konnte ich mittels STAT1-Immunpräzipitation acetyliertes STAT1 nachweisen. Für diese Beobachtung war eine recht lange IFN-Stimulation von 4 h nötig. Dies ist in Übereinstimmung mit einem Restimulierungsexperiment, bei welchem erst nach 4 h IFNγ durch eine erneute Kurzzeitstimulation für 20 min kein Phospho-STAT1 induzieren werden konnte [Ginter, 2012]. Es existiert demnach eine Phosphorylierungs-Latenzzeit wie sie auch für IFNα beschrieben wurde [Krämer, 2009].

Aufgrund der bekannten Dephosphorylierung von STAT1 durch TCP45 untersuchten wir erstmals mit Hilfe eines Förster-Resonanz-Energietransfers (FRET) die Interaktion zwischen STAT1 und TCP45. Nach dreistündiger Stimulation mit IFNα oder IFNγ ist zwischen beiden Proteinen eine starke Wechselwirkung im Zytoplasma zu verzeichnen. Dieses Ergebnis untermauert die Wichtigkeit der STAT1-Acetylierung für die anschließende Dephosphorylierung.

5.3 STAT1-Lysinmutanten bestätigen eine inaktivierende Wirkung der STAT1-Acetylierung

Die Acetylierung von Lysinresten durch HATs unterliegt einem dynamischen Gleichgewicht mit der Deacetylierung durch HDACs (siehe 2.2.1). Aufgrund dessen ist meist nur ein geringer Anteil eines betreffenden Proteins acetyliert. Dieser Anteil kann durch Verwendung von HDACi, Überexpression von HATs oder siRNA-vermittelten *knock down* von HDACs vergrößert werden. Um eine vollständige Acetylierung eines Lysinrests zu simulieren, macht man sich die strukturelle Ähnlichkeit zwischen Acetyllysin und Glutamin zu Nutze (Abbildung 6).

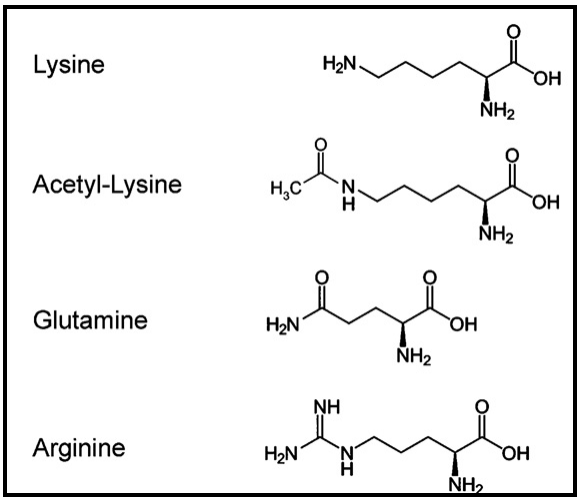


Abbildung 6: Strukturverwandtschaft einiger Aminosäuren mit Acetyllysin
Nach Acetylierung der Seitenkette ähnelt Lysin strukturell dem Glutamin. Arginin kann nicht acetyliert werden; aus [Krämer, 2010].

Der Austausch eines Lysins (K) zu Glutamin (Q) simuliert eine konstitutive Acetylierung, wogegen ein Arginin (R) eine Acetylierung der betreffenden Position ausschließt.
Aus vorherigen Publikationen unserer Arbeitsgruppe ging hervor, dass die Lysine K410 und K413 die mutmaßlichen Acetylierungsstellen in STAT1 sind [Krämer, 2006, Krämer, 2009]. In STAT1-negativen U3A Zellen ist es möglich STAT1-Acetylierungsmutanten ohne endogenen STAT1 Hintergrund zu analysieren.
Bei Verwendung einer entsprechenden Doppelglutaminmutante ($STAT1^{K410,413Q}$ – im folgenden $STAT1^{QQ}$ genannt) konnte in dieser Dissertation eine verminderte IFNγ-induzierte Tyrosin-701-Phosphorylierung beobachtet werden. Damit übereinstimmend konnte mit Hilfe von FRET eine im Vergleich zu Wildtyp STAT1 (STAT1wt) verstärkte Interaktion zwischen $STAT1^{QQ}$ und TCP45 detektiert werden [Ginter, 2012]. Dieses Ergebnis belegt zusätzlich eine acetylierungsgeleitete Dephosphorylierung von STAT1 durch TCP45. In folgenden Untersuchungen wurde mittels Fluoreszenzmikroskopie an lebenden Zellen nach IFNγ-Behandlung eine gestörte Translokation von $STAT1^{QQ}$ in den Nukleus aufgedeckt und in Zusammenhang damit eine beeinträchtigte

Transkriptionsaktivität verzeichnet. Weder ein STAT1 getriebener GAS-Luziferase-Reporter noch das endogene Zielgen UBCH8 konnten durch $STAT1^{QQ}$ induziert werden. Bemerkenswerterweise verhält sich die Doppelargininmutante ($STAT1^{K410,413R}$ – im folgenden $STAT1^{RR}$ genannt), das entsprechende Gegenstück zur $STAT1^{QQ}$, überaktiv. Das heißt $STAT1^{RR}$ ist sogar intensiver phosphoryliert als der Wildtyp und induziert auch einen GAS-Luziferase-Reporter stärker [Ginter, 2012]. $STAT1^{RR}$ bestätigt somit indirekt die hemmende Wirkung einer STAT1-Acetylierung.

5.4 IFNγ-induziertes phosphoryliertes $STAT1^{QQ}$ zeigt keine Transkriptionsaktivität

Die beschriebene stärkere Interaktion von TCP45 mit acetyliertem beziehungsweise pseudo-acetyliertem ($STAT1^{QQ}$) STAT1 führt zu einer schnelleren Dephosphorylierung [Krämer, 2009, Ginter, 2012].

Wir stellten uns die Frage, ob eine Wiederherstellung der Phosphorylierung durch Inhibition von Protein-Tyrosin-Phosphatasen (PTP) möglich wäre. Für diesen Zweck nutzten wir den universellen PTP-Inhibitor Orthovanadat. In der Tat konnten wir zeigen, dass bei 30 minütiger Vorinkubation mit Vanadat, IFNγ-induziertes phosphoryliertes-$STAT1^{QQ}$ (p-$STAT1^{QQ}$) gebildet wird. Aufgrund der Unspezifität von Orthovanadat nutzte ich in einem weiteren Versuch shRNA gegen TCP45, um die Expression dieser PTP herunterzuregulieren. In Kombination mit einer Überexpression der IFNGR-assoziierten Kinase JAK2 konnten wir so ebenfalls eine Phosphorylierung von $STAT1^{QQ}$ erreichen [Ginter, 2012].

Analog wurde in einer Vorarbeit unseres Labors p-$STAT1^{QQ}$ durch Inhibition der TCP45 und IFNα-Stimulation nachgewiesen. Dieses p-$STAT1^{QQ}$ ist funktionell aktiv und induziert ein STAT1-abhängiges GAS-Luciferasereporterkonstrukt und das endogene Zielgen UBCH8 [Krämer, 2009].

Bei der Untersuchung der Transkriptionsaktivität von durch IFNγ und Vanadat induziertem p-$STAT1^{QQ}$ mit Hilfe des GAS-Luciferasekonstrukts, konnte ich hingegen keine Aktivität messen. Eine Analyse der DNA-Bindungsfähigkeit mittels *Avidin-Biotin-Coupled DNA-Assay* (ABCD-Assay) ergab, dass $STAT1^{QQ}$ selbst in phosphorylierter Form nicht an GAS-enthaltende Oligonukleotide bindet [Ginter, 2012].

Im nächsten Schritt analysierte ich den Effekt den eine einzelne Lysinmutation auf das DNA-Bindeverhalten und auf die Transkriptionsaktivität hat. Sowohl $STAT1^{K410Q}$ als auch $STAT1^{K413Q}$ zeigten zwar eine stärkere Dephosphorylierung, konnten aber im Gegensatz zu $STAT1^{QQ}$ nach PTP-Inhibition an DNA binden und Transkription induzieren. Dieses Ergebnis lässt vermuten, dass die Anzahl an Acetylierungs-simulierenden Mutationen in der DBD einen entscheidenden Einfluss auf den IFNγ-induzierten Signalweg hat [Ginter, 2012].

Im Gegensatz zu Krämer et al. [Krämer, 2009], wo inaktives STAT1^{QQ} durch IFNα und gleichzeitige Phosphataseinhibition seine volle Funktionalität wiedererlangt, ist dies für IFNγ-induziertes p-STAT1^{QQ} nicht möglich. Dies zeigt, dass die inaktivierende STAT1-Acetylierung im Kontext mit dem jeweils induzierenden IFN gesehen werden muss.

5.5 Die Art des Zytokins bestimmt die Folgen der STAT1-Acetylierung

STAT-Proteine können durch eine Vielfalt von Zytokinen und Wachstumsfaktoren aktiviert werden (siehe 2.1.3). Häufig besitzt ein Ligand auch die Fähigkeit eine Phosphorylierung von mehreren verschiedenen STATs herbeizuführen [Schindler, 2008]. IFNα, ein Typ I IFN, bewirkt beispielsweise klassisch die Aktivierung von STAT1 und STAT2, welche zusammen mit IRF9 den ISGF3-Komplex bilden. Abhängig vom zellulären Kontext können aber auch andere STATs, wie STAT3 und STAT4 sowie seltener STAT5 und STAT6 durch IFNα phosphoryliert werden (Tabelle 2).

Tabelle 2: Aktivierung von bestimmten JAKs und STATs durch Zytokine aus der IFN- und gp130-Familie

Ligand	JAKs	STATs
Interferon-Familie		
IFN Typ I (z.B. IFNα,IFNβ)	**JAK1**, TYK1	**STAT1**, **STAT2**, STAT3, STAT4, (STAT5-6)
IFN Typ II (IFNγ)	**JAK1**, **JAK2**	**STAT1**
IFN Typ III (IFNλ)	JAK1, TYK2	**STAT1**, **STAT2**, STAT3
IL10	**JAK1**, **TYK2**	**STAT3**, STAT1
IL19	JAK1, JAK2	**STAT3**, STAT1
IL20	JAK1, JAK2	**STAT3**, STAT1
IL22 (IL-TIF)	JAK1, TYK2	**STAT3**, STAT1, (STAT5)
IL24 (mda7)	JAK1, JAK2	**STAT3**, STAT1
IL26 (AK155)	JAK1, TYK2	**STAT3**, STAT1
gp130-Familie		
IL6	**JAK1**, (JAK2)	**STAT3**, STAT1
IL11	JAK1	**STAT3**, STAT1
LIF	**JAK1**, (JAK2)	**STAT3**, STAT1
OSM	JAK1, (JAK2)	**STAT3**, STAT1
IL31	JAK1, (JAK2)	STAT3, STAT5, STAT1
G-CSF	JAK1, (JAK2)	**STAT3**
Leptin	JAK2	**STAT3**
IL12 (p35 + p40)	**TYK2**, JAK2	**STAT4**
IL23 (p19 + p40)	**TYK2**, JAK2	**STAT3**, **STAT4**, STAT1
IL27 (p28 + EBI3)	JAK2	**STAT1**, **STAT3**, STAT4, (STAT5)

Die Präferenzen der Zytokine für die JAK- und STAT-Aktivierung sind „***in fett"*** für sehr häufig, „normal" für selten und „(in Klammern*)"* für sehr selten aufgeteilt; IL – Interleukin; LIF – Leukemia Inhibitory Factor; OSM – Oncostatin M; G-CSF - Granulocyte-Colony Stimulating Factor; modifiziert nach [Schindler, 2008].

Im Gegensatz dazu leitet Typ II IFN (IFNγ) hauptsächlich eine Phosphorylierung von STAT1 ein. Diese Vorliebe von bestimmten Liganden für ausgewählte JAKs und STATs

beeinflusst die Bildung von Homo- oder Heterodimeren. So führt IFNγ ausschließlich zur Assoziierung von STAT1/STAT1 Homodimeren, wogegen für IFNα die Bildung von STAT1/STAT2/IRF9 und STAT1/STAT3 Heterodimeren sowie von STAT1, STAT3, STAT4, STAT5 und STAT6 Homodimeren möglich ist [Wesoly, 2007, Schindler, 2008].

Ich konnte in meiner Arbeit die gleichzeitige Tyrosinphosphorylierung von STAT1 und STAT3 nach IFNα-Inkubation bestätigen. In Zusammenhang damit war es auch möglich die Proteininteraktion von STAT1 und STAT3 mittels Immunpräzipitation zu detektieren sowie die Bindung beider Proteine an einem GAS-Oligonukleotid nachzuweisen. Diese gleichzeitige Bindung an DNA beziehungsweise die Interaktion beider STATs erfolgte jedoch nicht nach IFNγ-Stimulation [Ginter, 2012].

Wir fragten uns, ob der Dimerpartner somit das Schicksal von acetyliertem STAT1 bestimmt. Um möglichst klare Effekte zu beobachten, nutzten wir die Acetylierung-simulierende $STAT1^{QQ}$ Mutante, welche einer vollständigen Acetylierung des STAT1-Pools entspricht. In STAT1 negativen U3A Zellen konnten wir in einem ABCD-Assay die GAS-DNA-Bindung nur nach Vanadatvorbehandlung für IFNα-induziertes p-$STAT1^{QQ}$ nachweisen. IFNγ mit, sowie IFNα ohne Vanadatvorbehandlung genügen nicht, um eine DNA-Bindung von $STAT1^{QQ}$ zu ermöglichen. Gleichzeitig ließ sich nur bei IFNα plus Vanadat p-STAT3 auf dem Oligonukleotid detektieren [Ginter, 2012]. Dieses Ergebnis legt die Bildung eines p-STAT3/p-$STAT1^{QQ}$ Heterodimers nahe, bei welchem funktionell aktives STAT3 das beeinträchtigte $STAT1^{QQ}$ in einem „Huckepack“-Mechanismus an die DNA rekrutiert. Beide STATs müssen dafür tyrosinphosphoryliert sein, was eine Dimerbildung über SH2-Interaktionen vermuten lässt.

IL6 ist ein Zytokin der gp130-Familie, welches hauptsächlich STAT3 und zelltypabhängig seltener STAT1 aktiviert (Tabelle 2). In STAT1-rekonstituierten U3A Zellen findet jedoch ausschließlich eine Phosphorylierung von STAT3 statt [Ginter, 2012]. Wir nutzten U3A Zellen, welche transient mit $STAT1^{QQ}$ und STAT3wt transfiziert wurden, für einen *Electrophoretic-Mobility-Shift-Assay* (EMSA). An dem gleichen GAS-Oligonukleotid wie es für den ABCD-Assay verwendet wurde, konnte nach IL6 plus Vanadat-Stimulation ein Komplex beobachtet werden. Durch Zugabe von Antikörpern gegen STAT3 oder gegen eine an $STAT1^{QQ}$ klonierte und exprimierte Hämagglutinin-Sequenz (HA-tag) war es möglich die Laufstrecke des Komplexes zu verkürzen [Ginter, 2012]. Dies setzt eine spezifische Bindung der verwendeten Antikörper an den Komplex voraus und zeigt eine Beteiligung von STAT3 und STAT1 an, was wiederum die Bildung eines STAT3/STAT1 Heterodimers vermuten lässt.

5.6 Die Wahl des Zytokins reguliert die antivirale Aktivität von $STAT1^{QQ}$

IFNs vermitteln über STATs einen antiviralen Schutz, der zum angeborenen Immunsystem gehört. Die IFN-abhängige antivirale Aktivität wird routinemäßig durch Messung des durch Vesicular Stomatitis Virus (VSV) hervorgerufenen cytopathischen Effekts (CPE) bestimmt [Nusinzon, 2003, Tang, 2007].

Wir untersuchten die antivirale Aktivität von $STAT1^{QQ}$ im Vergleich zum Wildtyp nach IFNα- und IFNγ-Behandlung. Stabil STAT1wt exprimierende U3A-Zellen zeigten nach IFNα-Stimulation einen deutlichen Schutz vor VSV. Dieser war nach IFNγ-Behandlung etwas geringer, was in Übereinstimmung mit der Tatsache ist, dass der Großteil der antiviralen Gene durch IFNα-induziertes ISGF3 abgerufen wird. Im Vergleich zum Wildtyp besitzen stabil $STAT1^{QQ}$ exprimierende Zellen eine verringerte antivirale Aktivität. Interessanterweise ist $STAT1^{QQ}$ nach IFNα -Stimulation jedoch deutlich potenter als nach IFNγ-Stimulation [Ginter, 2012]. Diese Daten sind in Übereinstimmung mit der Beobachtung, dass inaktives acetyliertes STAT1 durch intakte Heterodimerpartner in seiner Funktion wiederhergestellt werden kann (siehe 5.5).

5.7 Die STAT1-Aktivität wird durch ein Phosphorylierungs-/ Acetylierungswechselspiel bestimmt

Um exzessive Zytokinstimulation zu verhindern muss die STAT1-Aktivität akkurat und angemessen reguliert werden. Der Einfluss von HDACi, HDAC *knock down* oder HAT-Überexpression hat Hinweise auf einen hemmenden Effekt von Acetylierung auf den STAT1-vermittelten Signalweg erbracht [Genin, 2003, Klampfer, 2003, Nusinzon, 2003, Chang, 2004]. Eine direkte Acetylierung von STAT1 kann durch verschiedene Stimuli wie beispielsweise IFNs, Lipopolysaccharid, Cisplatin, HDACi oder Alloantigene hervorgerufen werden. Der Großteil der Publikationen beschreibt dabei eine inaktivierende Wirkung der STAT1-Acetylierung [Wieczorek, 2012].

Unser Labor konnte einen STAT1 Aktivierungs-/Inaktivierungszyklus nachweisen, der für das Gleichgewicht der STAT1-Signaltransduktion entscheidend ist. Dabei translozíert p-STAT1 in den Nukleus, wo es wahrscheinlich nach DNA-Bindung durch die HAT CBP acetyliert wird. Acetyl-STAT1 verlässt die DNA und interagiert verstärkt mit der Phosphatase TCP45, was zu einer Dephosphorylierung führt [Krämer, 2009, Ginter, 2012]. Die Inaktivierung wird also durch die Acetylierung eingeleitet und die Acetylierung wird im Zytosol durch HDACs entfernt, womit STAT1 wieder für einen neuen Zyklus zur Verfügung steht. Dieser Phosphorylierungs/Acetylierungs-Schalter zeigt eindrucksvoll wie sich posttranslationale Modifikationen gegenseitig beeinflussen können.

Durch die Verwendung einer import-defizienten STAT1^{L407A,L409A} Mutante sowie einer tyrosinphosphorylierungs-defizienten STAT1^{Y701F} Mutante konnten wir zeigen, dass Kernimport und Phosphorylierung Voraussetzungen für die Acetylierung von STAT1 sind [Ginter, 2012]. Diese Erkenntnisse sind im Einklang mit dem von uns postulierten Phosphorylierungs/Acetylierungs-Schalter. Unklar bleibt, ob die Acetylierung von STAT1 durch CBP die Bindung von STAT1 an die DNA voraussetzt. Meyer et al. kreierten eine STAT1-Mutante, bei der an bestimmten mit DNA interagierenden Aminosäurepositionen (V426 und T427) Austausche durch negativ geladene Asparaginsäure durchgeführt wurden [Meyer, 2003]. Diese STAT1-DNA^{minus} genannte Mutante wird zwar tyrosinphosphoryliert bindet aber kaum DNA. Versuche mit STAT1-DNA^{minus} könnten in Zukunft Aufschluss darüber geben ob die DNA-Bindung eine Voraussetzung für Acetylierung von STAT1 ist.

Die vorliegende Dissertation zeigt auch, dass der Einfluss des stimulierenden Zytokins in das Modell mit aufgenommen werden muss. Je nach Induktion von acetyliertem STAT1 durch IFNα oder IFNγ ergeben sich andere Möglichkeiten der Dimerbildung (Abbildung 7). Anhand von konstitutiver Acetylierung simulierendem STAT1^{QQ} wurde deutlich, dass im Falle von IFNα ein Heterodimerpartner das inaktive STAT1^{QQ} funktionell wiederherstellen kann. Dieser Heterodimerpartner kann beispielsweise STAT1, STAT2 oder STAT3 sein [Ginter, 2012]. Auf der anderen Seite werden durch IFNγ nur STAT1-Homodimere gebildet, wodurch STAT1^{QQ} inaktiv bleibt.

Interessant wäre die Überprüfung dieses Prinzips durch andere Zytokine, die ähnlich wie IFNα, mehrere STATs aktivieren können. IL27 induziert beispielsweise eine Phosphorylierung von STAT1 und STAT3 sowie in geringerem Ausmaß p-STAT4 und p-STAT5 (siehe Tabelle 2). Auch Kombinationen verschiedener Zytokine könnten bei inaktivem STAT1^{QQ} Wildtyp-Funktionen gewähren. Diese Bedingungen kommen zudem der Situation *in vivo* näher, in welcher oft Zytokincocktails auf Zellen wirken und so eine Feinabstimmung verschiedener Prozesse erlauben.

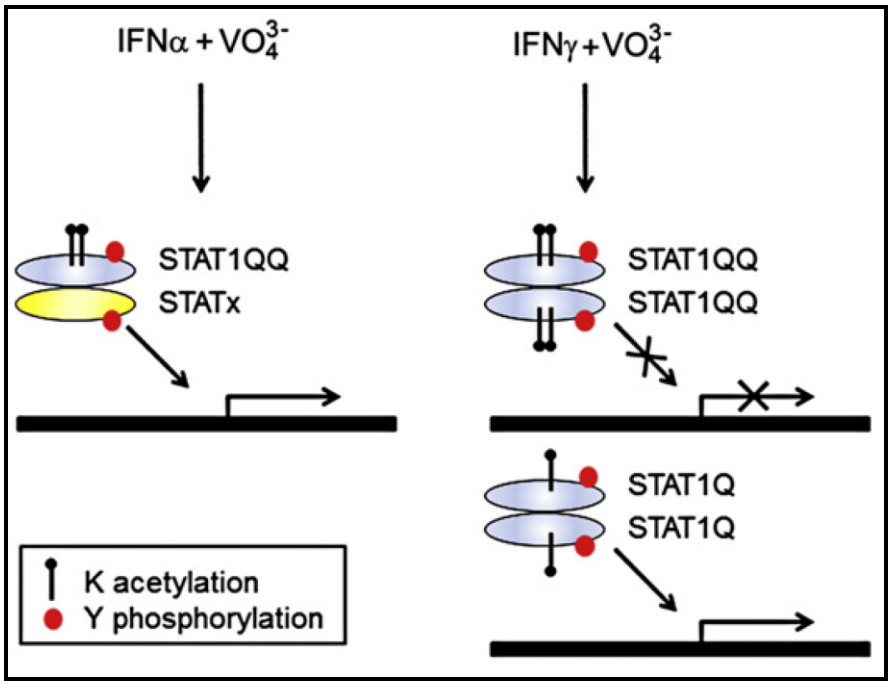


Abbildung 7: Die Art des Interferons differenziert die biologische Auswirkung der STAT1-Acetylierung

Die Acetylierung von STAT1 hat einen hemmenden Effekt auf die STAT1-Aktivität. Abhängig vom IFN kann die Funktion von Acetylierung-simulierendem $STAT1^{QQ}$, im Zusammenhang mit einer Vanadat vermittelten PTP-Inhibition, wiederhergestellt werden. IFNα bewirkt die Phosphorylierung verschiedener STATs und erlaubt die erfolgreiche Rekrutierung des inaktiven $STAT1^{QQ}$ im Heterodimer mit einem funktionellen STAT (STATx) an die DNA. IFNγ hingegen induziert ausschließlich Homodimere aus $STAT1^{QQ}$, die inaktiv bleiben. Einzelne, durch Glutamin (Q) simulierte Acetylierungen an Lysin 410 oder 413 genügen nicht, um STAT1 bei forcierter Phosphorylierung durch IFNγ und Vanadat zu inaktivieren; nach [Ginter, 2012].

Eine funktionelle Wiederherstellung von inaktivem STAT1 durch andere STATs konnten auch Sadzak und Mitarbeiter beobachten. Die Serinphosphorylierung von STAT1 setzt eine DNA-Bindung voraus. Mit Hilfe einer DNA-Bindungs-defizienten STAT1-Mutante ($STAT1^{L407A}$) wurde gezeigt, dass STAT2 und IRF9 die DNA-Bindung dieser Mutante vermitteln können. Dies war jedoch nur nach IFNβ und nicht nach IFNγ möglich, da IFNβ die Bildung von ISGF3 ermöglicht und IFNγ nur STAT1-Homodimere induziert [Sadzak, 2008].

5.8 Das STAT1 Acetylierungsmodul ist übertragbar

Eine Acetylierung von STAT1 wurde von uns und anderen Forschergruppen nachgewiesen [Krämer, 2006, Guo, 2007, Hayashi, 2007, Tang, 2007, Cudejko, 2011, Stronach, 2011, Banik, 2012]. Wir konnten deutliche Hinweise auf die Acetylierungspositionen K410 und K413 liefern, welche in der DBD liegen [Krämer, 2009]. In unabhängigen MS-Analysen wurden diese Acetyllysinreste später bestätigt [Wieczorek]. Bemerkenswerterweise ist die DBD bei allen STATs konserviert und bis auf STAT2 und STAT3 tragen alle STATs Lysine analog zu K410 und K413 (Abbildung 8). Die DBDs von STAT1 und STAT3 weisen sogar

circa 80 % Aminosäuresequenzhomologie auf, unterscheiden sich jedoch an den genannten Lysinen 410 und 413. An den entsprechenden Positionen trägt STAT3 zwei Argininreste (R414 und R417) [Melen, 2001].

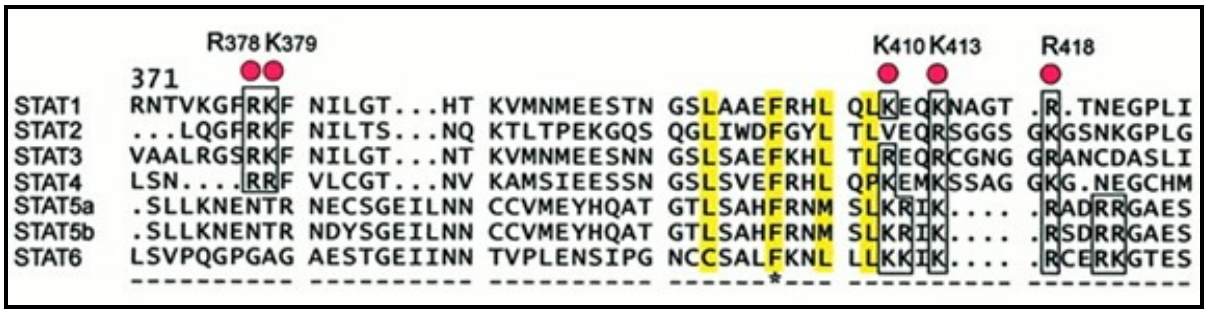


Abbildung 8: Aminosäuresequenzvergleich der STAT-DBD
Die DBD ist in allen STATs gut konserviert. STAT1 und STAT3 zeigen besonders große Homologie, unterscheiden sich jedoch in den Positionen 410 und 413 voneinander. Aus [Melen, 2001]

Wir stellten uns die Frage, ob die Übertragung des STAT1-Acetylierungsmotivs auf STAT3 die Eigenschaften dieses Proteins analog verändern wird. Zusätzlich untersuchten wir dieses Konzept an überaktiven NTD-Mutanten von STAT1.

5.8.1 STAT3 DBD-Mutanten zeigen eine reduzierte Phosphorylierung

Der Austausch der Arginine von STAT3 an Positionen 414 und 417 zu Lysinen führt zu einer Situation wie sie in STAT1 vorzufinden ist. Folglich wären Acetylierungen an diesen in der DBD lokalisierten Stellen in STAT3 denkbar. Das Ersetzen der Arginine durch Glutamine würde einer konstitutiven Acetylierung gleichkommen, da Acetyllysin- und Glutaminreste strukturell ähnliche Seitenketten aufweisen (Abbildung 6).
In der STAT3- und STAT5-negativen Zelllinie PC3 lassen sich Einflüsse von STAT3-Mutanten ohne Beeinflussung durch endogenes STAT3 und STAT5 untersuchen. Eine Doppelmutante von STAT3, in welcher die Lysine 414 und 417 durch Glutamine ersetzt wurden ($STAT3^{R414,417Q}$), konnte nur sehr schlecht exprimiert werden. Aus diesem Grund wurden die Einzelglutaminmutanten, $STAT3^{R414Q}$ und $STAT3^{R417Q}$ untersucht. Für die $STAT3^{R414Q}$ Mutante konnten stärkere Effekte auf die Tyrosinphosphorylierung nach IL6-Stimulation beobachtet werden als für $STAT3^{R417Q}$, weshalb die anschließenden Versuche mit $STAT3^{R414Q}$ durchgeführt wurden. $STAT3^{R414Q}$ weist im Vergleich zum Wildtyp reduzierte p-STAT3 Spiegel auf. Die Phosphorylierung lässt sich jedoch durch Verwendung des Breitband-PTP-inhibitors Orthovanadat wiederherstellen. Immunfluoreszenz-Mikroskopie von stabil exprimierenden PC3-Zellen zeigte für $STAT3^{R414Q}$, in Übereinstimmung mit der reduzierten Phosphorylierung, eine fehlende Translokation in den Zellkern nach IL6-Stimulation. Vanadat und die spezifische shRNA gegen TCP45 erlaubten die Phosphorylierung von $STAT3^{R414Q}$ und damit den Eintritt in den Nukleus [Ginter, in

Vorbereitung]. Dass TCP45 STAT1 und STAT3 dephosphorylieren kann, ist bekannt und unterstreicht den Einfluss der strukturellen Ähnlichkeit beider STATs [Yamamoto, 2002].
Unter Verwendung einer Doppel-Lysinmutante STAT3^{R414,417K} (im Folgenden STAT3^{KK}) konnte ebenfalls eine geringere Phosphorylierung im Vergleich zum Wildtyp nachgewiesen werden [Ginter, in Vorbereitung].
Die Ergebnisse für STAT3^{R414Q} und STAT3^{KK} erinnern an die Situation wie sie für acetyliertes STAT1 bekannt ist. Acetyl-STAT1 oder auch pseudo-acetyliertes STAT1^{QQ} interagiert nachweislich stärker mit TCP45, was zu einer Dephosphorylierung und damit Inaktivierung von STAT1 führt [Krämer, 2009, Ginter, 2012].

5.8.2 Untersuchung der Transkriptionsaktivität der STAT3 DBD-Mutanten

Im nächsten Schritt sollten mittels ABCD- und Luziferase-Assay der Einfluss des veränderten Phosphorylierungsverhaltens der STAT3-Mutanten auf die DNA-Bindung und Transkriptionsaktivierung analysiert werden. Zu diesem Zweck wurden Luziferasereporterkonstrukte verwendet, die in ihrem Promotor GAS- oder SIE-Bindungselemente besitzen (siehe 2.1.3). GAS-Sequenzen erlauben hauptsächlich die Bindung von STAT1-Homodimeren, aber auch STAT3/STAT3 und STAT1/STAT3 Dimere können binden. SIE-Sequenzen rekrutieren überwiegend Homodimere, die aus STAT3 bestehen (Abbildung 9).

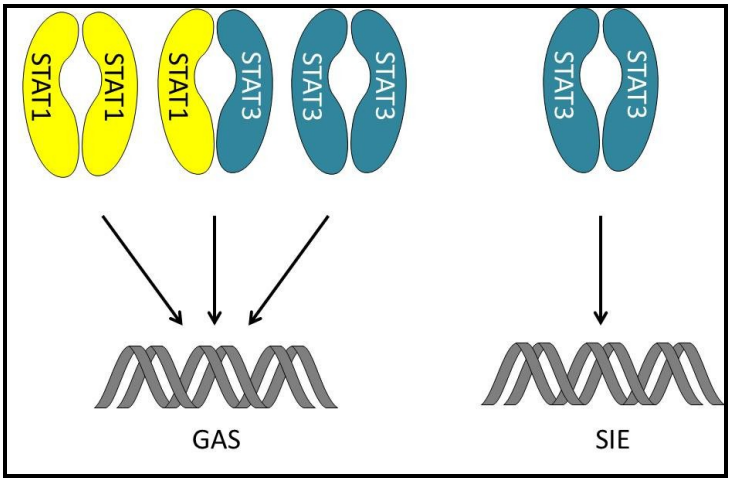


Abbildung 9: Bindung unterschiedlicher STAT-Dimere an verschiedene DNA-Bindungssequenzen
Promotoren mit GAS-Bindungselementen rekrutieren vornehmlich Dimerkombinationen aus STAT1 und STAT3. SIE-Sequenzen erlauben überwiegend die Bindung von STAT3 Homodimeren. U3A-Zellen (STAT1-/-) ermöglichen die Analyse von STAT3-Homodimeren an GAS-Elementen und PC3-Zellen (STAT3-/-, STAT5-/-) gestatten die Untersuchung von transient exprimierten STAT3-Mutanten ohne Beeinflussung durch endogenes STAT3 und STAT5.

ABCD-Assays deckten in PC3 Zellen eine beeinträchtigte GAS-DNA Bindung von $STAT3^{R414Q}$ auf, welche jedoch durch PTP-Inhibition kompensiert werden konnte. In Übereinstimmung damit zeigte $STAT3^{R414Q}$ auch eine verminderte Induktion eines STAT3-abhängigen SIE-Luziferase-Reporters. Am selben Reporter war auch $STAT3^{KK}$ nicht fähig IL6-induzierte SIE-Luziferase-Transkription hervorzurufen [Ginter, in Vorbereitung].
Wir überprüften weiterhin eine STAT3-abhängige Aktivierung eines GAS-Luziferase-Reporters in STAT1 negativen U3A-Zellen. Auch in diesem System ließ sich nach IFNα oder IL6-Stimulation eine STAT3-vermittelte Aktivierung beobachten. Da IL6 in U3A-Zellen spezifisch STAT3 aktiviert, kommen für die Steigerung des GAS-Luziferase-Signals nur Homodimere aus STAT3 in Frage. Im Einklang mit den Daten des SIE-Luziferase-Reporters zeigen $STAT3^{R414Q}$ und $STAT3^{KK}$ auch eine verminderte Aktivierung des GAS-Luziferase-Reporters [Ginter, in Vorbereitung].
Die Einführung eines STAT1-Acetylierungsmoduls in STAT3 bewirkt anscheinend eine Hemmung der STAT3 Transkriptionsaktivität. Wir fragten uns, wie HDACi auf zusätzlich eingeführte Lysinreste in der DBD wirken und auf welche Weise folglich $STAT3^{KK}$ beeinflusst wird. Der pan-HDACi TSA wurde mit IFNα angewendet und führte zu einer erhöhten Aktivität von STAT3wt auf dem GAS-Luziferase-Reporter. $STAT3^{KK}$ hingegen, zeigte keine gesteigerte Aktivität bei Kostimulation [Ginter, in Vorbereitung]. Dies ist zunächst mit dem generell positiven Einfluss der Acetylierung auf die Transkriptionsaktivität von STAT3 zu erklären. Sowohl für die NTD als auch für den C-terminalen Bereich von STAT3 sind aktivierende Acetylierungen beschrieben [Wieczorek, 2012]. Der Austausch der Arginine 414 und 417 zu Lysinen in der STAT3 DBD generiert zusätzliche potentielle Acetylierungsstellen und bewirkt eine Kompensierung der zuvor aktivierenden Acetylierung. Folglich ist ein negativer Effekt der neugeschaffenen Lysine zu vermuten, der wahrscheinlich durch Acetylierung vermittelt wird. Dies impliziert, dass inaktivierende Eigenschaften der STAT1-Acetylierung auf STAT3 übertragbar sind.

5.8.3 Der Einfluss von $STAT3^{R414Q}$ auf endogene Zielgene

Nachdem die Transkriptionsaktivität der STAT3-DBD-Mutanten von uns ausführlich anhand von Luziferase-Reportersystemen studiert wurde, interessierte uns die Regulation endogener Zielgene.
Die E2-Ubiquitin-Konjugase UBCH8 wird durch Typ I sowie Typ II IFN induziert und dieser Prozess ist durch GAS und ISRE Promotorbindestellen vermittelt [Nyman, 2000, Krämer, 2009]. Da GAS-Bindungselemente auch durch STAT3 gebunden werden können [Timofeeva, 2012a], untersuchten wir ob eine Regulation durch diesen Transkriptionsfaktor möglich ist.

Ein *knock down* von STAT3 in mit STAT1 rekonstituierten U3A-Zellen führte zu einer Reduktion von durch IFNα induziertem UBCH8 [Ginter, in Vorbereitung]. Dies legt eine positive Wirkung von STAT3 auf die UBCH8-Expression nahe. Wir fragten uns, wie $STAT3^{R414Q}$ die UBCH8-Proteinspiegel beeinflusst. Nach transienter Überexpression von $STAT3^{R414Q}$ in STAT1 rekonstituierten U3A-Zellen verzeichneten wir eine Verminderung von UBCH8 im Vergleich zu STAT3wt. Ebenso wurde das STAT3-Zielgen PIM1 reduziert exprimiert. In stabil $STAT3^{R414Q}$ exprimierenden PC3-Zellen konnte eine verringerte UBCH8-Expression bestätigt werden [Ginter, in Vorbereitung].
Diese Ergebnisse sind im Einklang mit den mittels Luziferase-Assay gewonnenen Daten und zeigen, dass STAT3 an der Regulation der UBCH8-Expression beteiligt ist. Möglicherweise wird dies durch STAT1/STAT3 Heterodimere vermittelt. Eine Bildung solcher Dimere nach IFNα-Stimulation ist bekannt, jedoch liegen nur wenige Daten zu einer STAT1/STAT3 Zielgenregulierung vor [Ho, 2006, Schiavone, 2011]. $STAT3^{R414Q}$, welches ein STAT1-Acetylierungsmodul trägt, verliert die Fähigkeit zur Regulation der UBCH8- und PIM1-Expression.
STAT1 und STAT3 sind strukturell nah verwandt, vermitteln jedoch oft gegensätzliche Prozesse. Zudem unterliegt STAT3 einem aktivierenden Einfluss durch Acetylierung, wogegen für Acetyl-STAT1 eine inhibierende Wirkung beschrieben ist [Wieczorek, 2012]. STAT1 und STAT3 sind evolutionär aus einem Vorfahren hervorgegangen [Wang, 2012]. Der evolutionäre Prozess könnte die Acetylierung der DBD als ein wichtiges Unterscheidungsmerkmal zur Regulation beider STATs hervorgebracht haben. Eine Übertragung des STAT1 Acetylierungsmoduls auf STAT3 durch Einführung von Punktmutationen an Position 414 und 417 transferiert auch die hemmenden Eigenschaften der STAT1-Acetylierung. Wir kreierten STAT3-Mutanten, die so nicht *in vivo* existieren, um das Prinzip der STAT1-Acetylierung durch Übertragbarkeit zu bestätigen.

5.8.4 Eine konstitutive Acetylierung inaktiviert N-terminale STAT1-Mutanten

Die NTD von STAT-Proteinen vermittelt Wechselwirkungen zwischen unphosphorylierten STATs und reguliert die Tetramerisierung beziehungsweise Oligomerisierung. Des Weiteren ist sie entscheidend für die Inaktivierung, denn in der antiparallelen Dimerkonformation ist das phosphorylierte Tyrosin zur Außenseite gerichtet, was eine leichtere Dephosphorylierung erlaubt (siehe 2.1.4 und Abbildung 3).
Ein Austausch des Phenylalanins 77 und des Leucins 78 durch Alanine in der STAT1-NTD ($STAT1^{AA}$) bewirkt eine Störung der NTD-vermittelten Dimerisierung. Daraus resultiert ein STAT1-Molekül, welches einen monomeren Charakter aufweist. Aus der Literatur ist

zusätzlich bekannt, dass $STAT1^{AA}$ länger als der Wildtyp phosphoryliert bleibt [Zhong, 2005, Mertens, 2006].

Wir beobachteten für $STAT1^{AA}$ ebenfalls eine verzögerte Dephosphorylierung und konnten zudem eine überaktive Transkriptionsinduktion zeigen [Ginter, in Vorbereitung].

In der Theorie sollte eine verstärkte Acetylierung von $STAT1^{AA}$ eine erleichterte TCP45-Interaktion und folglich eine Inaktivierung von STAT1 zur Folge haben. Uns war es nicht möglich eine Acetylierung von $STAT1^{AA}$ nach HDACi- und IFN-Behandlung mittels Immunpräzipitation nachzuweisen [Ginter, in Vorbereitung]. Darum führten wir Lysin zu Glutamin Mutationen in $STAT1^{AA}$ ein und erhielten so eine simuliert-konstitutive Acetylierung an den beschriebenen Stellen 410/413 ($STAT1^{AA/QQ}$) [Krämer, 2006, Ginter, in Vorbereitung]. Dieses nun durchgängig aktivierte STAT1-Acetylierungsmodul in $STAT1^{AA/QQ}$ bewirkte interessanterweise eine Aufhebung der Überaktivität von $STAT1^{AA}$. $STAT1^{AA/QQ}$ wird kaum tyrosinphosphoryliert und vermittelt in Übereinstimmung damit keine Transkriptionsaktivität auf einem GAS-Luziferasereporter sowie keine Expression des endogenen Zielgens UBCH8 [Ginter, in Vorbereitung]. Erstaunlicherweise konnte die Phosphorylierung von $STAT1^{AA/QQ}$ durch Vorbehandlung der Zellen mit Orthovanadat wiederhergestellt werden [Ginter, in Vorbereitung]. Dieser Inhibitor für PTPs hemmt auch TCP45, welche für die STAT1-Dephosphorylierung verantwortlich ist [ten Hoeve, 2002].

Wie für STAT1wt zeigt sich auch für die NTD-Mutante $STAT1^{AA/QQ}$ eine negative (pseudo)-acetylierungsabhängige Regulation durch Phosphatasen wie TCP45 [Krämer, 2009, Ginter, 2012]. Wir konnten somit nachweisen, dass eine Dimerisierungsfähigkeit nicht erforderlich ist, um die hemmenden Eigenschaften des Acetylierungsmoduls zu vermitteln. Die dauerhaft phosphorylierte NTD-Mutante $STAT1^{AA}$ unterliegt einer mangelhaften Kontrolle durch TCP45. Durch Einführung von Acetylierung-simulierenden Glutaminresten in $STAT1^{AA}$ wird $STAT1^{AA/QQ}$ kreiert, welches hierdurch einer Regulation durch Dephosphorylierung wieder zugänglich ist. Abbildung 10 gibt eine Übersicht zur Phosphorylierungsfähigkeit der in Kapitel 5.8 beschriebenen STAT1- und STAT3-Mutanten; die in [Ginter, in Vorbereitung] verwendet wurden.

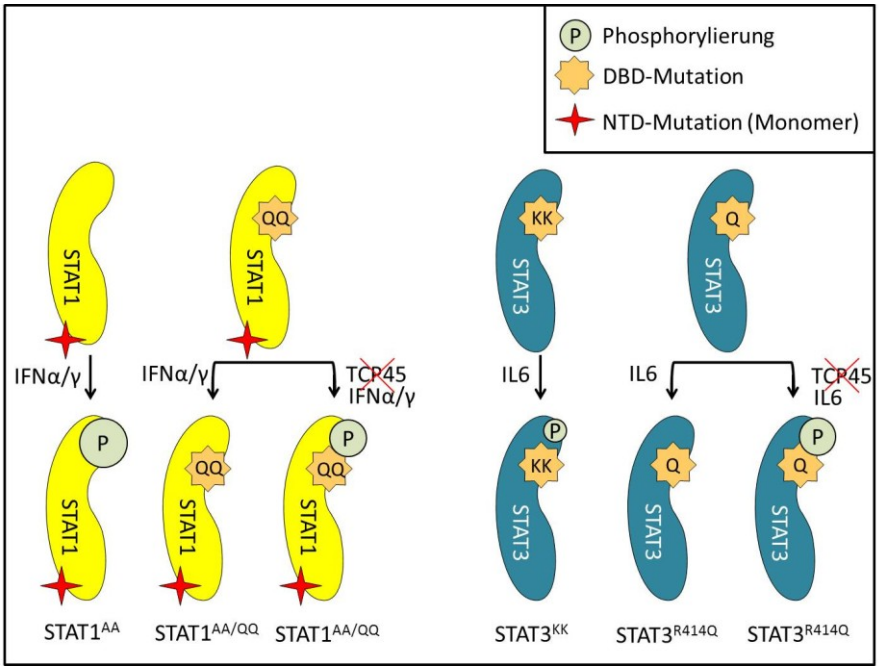


Abbildung 10: Phosphorylierungsstatus von STAT1-DBD/-NTD-Mutanten und von STAT3-DBD-Mutanten

Eine Mutation in der NTD von STAT1 vermittelt monomeren Charakter und erlaubt durch IFNα/γ-Stimulation eine anhaltende Tyrosinphosphorylierung ($STAT1^{AA}$). Zusätzliche Mutationen in der DBD zu Acetylierung simulierenden Glutaminen verhindern eine STAT1-Phosphorylierung ($STAT1^{AA/QQ}$). Die Hemmung der Phosphatase TCP45 ermöglicht jedoch die Phosphorylierung von $STAT1^{AA/QQ}$. STAT3-DBD-Mutanten der zu STAT1 korrespondierenden Aminosäuren zeigen eine verminderte Phosphorylierung ($STAT3^{KK}$) oder keine Phosphorylierung ($STAT3^{R414Q}$) nach Stimulation mit IL6. $STAT3^{R414Q}$ lässt sich jedoch nach Hemmung von TCP45 und gleichzeitiger IL6-Stimulation phosphorylieren. Die Größe der Markierung für Phosphorylierung in der Abbildung gibt Auskunft über das Ausmaß beziehungsweise die Kinetik der Phosphorylierung. Legende oben rechts.

5.8.5 $STAT1^{AA/QQ}$ unterliegt dem inhibierenden Einfluss des Acetylierungsmoduls

Wir untersuchten, ob die Phosphorylierung von $STAT1^{AA/QQ}$ die biologische Funktion dieser Mutante wiederherstellen kann. Die Transkriptionsfähigkeit wurde anhand eines STAT1-abhängigen Luziferasereporters ermittelt. Phosphoryliertes $STAT1^{AA/QQ}$ ($p\text{-}STAT1^{AA/QQ}$) konnte diesen Reporter nicht aktivieren. Übereinstimmend damit war $p\text{-}STAT1^{AA/QQ}$ ebenso unfähig im ABCD-Assay an GAS-Sequenzen zu binden. Dabei war es unerheblich, ob mit IFN Typ I oder II stimuliert wurde [Ginter, in Vorbereitung]. Dies legt nahe, dass $STAT1^{AA/QQ}$ aufgrund seiner N-terminalen Mutation, nicht in der Lage ist, einen kompetenten Heterodimerpartner zu rekrutieren, der die inaktivierende Wirkung der pseudo-Acetylierung aufheben kann. Für IFNα-aktiviertes $STAT1^{QQ}$ wurde durch unsere Gruppe solch ein Mechanismus bereits beschrieben [Krämer, 2009, Ginter, 2012]. Des Weiteren konnte im angeführten ABCD nach IFNα und Vanadat Kostimulation auch p-STAT3 auf dem GAS-Oligonukleotid nachgewiesen werden, welches ein potentieller Heterodimerpartner ist.

Eine Immunpräzipitation gegen $STAT1^{AA}$ und $STAT1^{AA/QQ}$ zeigte, dass im Vergleich zum Wildtyp beide NTD-Mutanten, selbst nach IFN-Stimulation, nicht mit STAT2 interagieren

[Ginter, in Vorbereitung]. Interessanterweise sind NTD-Interaktionen als homotypisch beschrieben [Ota, 2004]. Das heißt sie werden nur zwischen gleichen STAT-Proteinen ausgebildet. Wir beobachteten jedoch eine STAT1/STAT2 Interaktion im unstimulierten Zustand, die nur beim Wildtyp und nicht bei den NTD-Mutanten auftrat, was erstmals einen Einfluss der NTD auf die Heterodimerbildung belegt.

Im nächsten Schritt interessierte uns die Fähigkeit der NTD-Mutanten zur Vermittlung eines antiviralen Schutzes. Dazu wurde der durch VSV induzierte CPE gemessen. Stabile U3A-Zellen mit $STAT1^{AA/QQ}$ zeigten einen geringeren Schutz vor VSV als jene mit $STAT1^{AA}$, was mit der inaktivierenden pseudo-Acetylierung zu erklären ist. Übereinstimmend mit dem Ergebnis der Immunpräzipitation, können $STAT1^{AA}$ und $STAT1^{AA/QQ}$ keine verbesserte antivirale Abwehr nach IFNα-Stimulation gewährleisten [Ginter, in Vorbereitung]. Beide wiesen eine im Vergleich zu STAT1wt geringe Interaktion mit STAT2 auf, welches jedoch im Komplex mit IRF9 und STAT1 für die Expression eines Großteils der antiviralen Gene notwendig ist [Tang, 2007].

5.9 Der Kernimport der verwendeten DBD-Mutanten ist nicht per se defekt

Die verwendeten Mutanten machen deutlich, dass Punktmutationen in der STAT1- oder STAT3-DBD nicht zum generellen Defekt in der Signalübermittlung führen. Die STAT1 Lysine K410 und K413 gehören zum Kernlokalisierungssignal (NLS). Eine Alaninmutante der betreffenden Stellen ($STAT1^{K410,413A}$) ist zwar phosphorylierbar, zeigt aber keine Interaktion mit Importin α5, was in defektem Kernimport resultiert [Fagerlund, 2002]. Diese Beobachtung ist jedoch unter Berücksichtigung eines Verlusts der positiven Ladung durch Lysin zu Alanin Mutation zu sehen, welche die Konformation und Interaktion mit Importin α5 entscheidend verändern kann und gleichzeitig eine Lysinacetylierung ausschließt. Weiterhin konnte durch einen einzelnen Austausch des Leucins 407 zu Alanin und durch zusätzliche Leucin 409 zu Alanin Mutation, ebenfalls eine Interaktion mit Importin α5 verhindert werden [Reich, 2006, Sadzak, 2008]. Dies zeigt, dass auch andere Positionen den Kernimport vermitteln. Unsere Daten zeigen, dass Mutationen von K410/K413 zu Arginin ($STAT1^{RR}$) oder Glutamin ($STAT1^{QQ}$) immer noch die Kerntranslokation erlauben [Krämer, 2009, Ginter, 2012].

Der Kernimport von STAT3 wird durch die Importine α5 und α7 vermittelt. Die Argininreste R214/R215 und R414/R417 von STAT3 sind notwendig, um die Interaktion mit diesen Importinen zu gewährleisten. Dabei haben R214/R215 einen essentiellen Einfluss auf die Importinbindung, was verkürzte STAT3-Varianten eindrucksvoll zeigen. R414/R417 hingegen sind vermutlich für die Vermittlung der richtigen Konformation nötig [Ma, 2003,

Ma, 2006]. Alaninmutanten beider Paare resultieren in einer defekten Kerntranslokation [Ma, 2003]. In der vorliegenden Arbeit konnte jedoch beobachtet werden, dass $STAT3^{R414Q}$ nach PTP Inhibition zum Kernimport fähig ist [Ginter, in Vorbereitung]. Dies kann dadurch begründet sein, dass Alanin strukturell unähnlicher zu Arginin ist als Glutamin, oder dass das verbleibende Arginin 417 in $STAT3^{R414Q}$ für die Vermittlung der Importininteraktion ausreicht.

Mutationen in der DBD führen nicht zwangsläufig zu einem generellen Funktionsverlust. STAT1- und STAT3-Mutanten lieferten Beweise für eine phosphorylierungsabhängige Regulation der Aktivität durch Lysin- und Argininreste, wobei die Funktionalität der DBD erhalten bleibt. Daten aus *Caenorhabdites elegans* belegen, dass das STAT-Äquivalent STA-1 nicht immer positiv in die STAT-Aktivierung eingebunden ist [Wang, 2006] und geben somit einen Hinweis für eine evolutionär frühe Etablierung eines Regulationsmechanismus durch die DBD.

5.10 HDACi erlauben STAT1- und STAT3-Acetylierung als Therapieansatz

HDACi werden in laufenden klinischen Studien verwendet und zeigten bereits Erfolge bei der Behandlung von Krebs- und Autoimmunerkrankungen sowie bei der Verhinderung von mit Transplantation verbundenen immunologischen Abstoßungsreaktionen [Leng, 2006, Müller, 2010, Delcuve, 2012]. Die genauen Wirkmechanismen der HDACi und die Beziehungen zwischen HDACs und Nicht-Histon-Proteinen sind nicht vollständig aufgeklärt. HDACi hemmen oft verschiedene HDAC ähnlich gut und zusätzlich kann jede HDAC mehrere Substrate deacetylieren [Spange, 2009, Ginter, 2013]. Für alle Mitglieder der STAT-Proteinfamilie wurden bereits Acetylierungen nachgewiesen, die biologische Funktionen zum Teil entscheidend verändern können [Wieczorek, 2012].

STAT1 reguliert sowohl die angeborene als auch die erworbene Immunität und kontrolliert das Zellwachstum [Mertens, 2007, Hiscott, 2011, Gough, 2012]. Der Großteil der Literatur bestätigt einen hemmenden Einfluss der Acetylierung auf STAT1-vermittelte Signalwege, welcher durch eine beschleunigte Dephosphorylierung von STAT1 bewirkt wird [Wieczorek, 2012]. Im Gegensatz dazu hat die Acetylierung einen aktivierenden Einfluss auf STAT3. Da überaktives STAT3 in vielen Krebsarten eine wichtige Rolle spielt, ist die wachsende Anzahl an Studien, in welchen HDACi der Tumorprogression entgegenwirken, zunächst verwunderlich [Groner, 2008, Han, 2010, Hubaux, 2010, Müller, 2010, Tang, 2010].

Eine Erklärung dafür könnte die Beeinflussung verschiedener Signalwege und deren Vernetzung durch HDACi-vermittelte Acetylierung sein. Acetylierung kann beispielsweise die Proteinstabilität, die Lokalisation und die DNA-Bindung verändern, kann aber auch neue

Proteininteraktionen ermöglichen [Spange, 2009]. Acetyliertes STAT1 interagiert etwa verstärkt mit NF-κB und bewirkt seinen Export aus dem Zellkern, wodurch anti-apoptotische Gene nicht weiter transkribiert werden [Krämer, 2006]. Zusätzlich kann STAT1 in Wechselwirkung mit p53 Apoptose und Seneszenz induzieren [Kim, 2007]. Solche Zusammenspiele könnten ebenso durch Acetylierung reguliert werden. Auch STAT3 interagiert mit den krebsrelevanten Proteinen NF-κB und p53. Wie diese Wechselwirkungen durch HDACi moduliert werden können, bleibt ein interessantes Forschungsfeld [Wieczorek, 2012].

Fehlreguliertes STAT1 kann Immunreaktionen stören und Autoimmunerkrankungen begünstigen. Ein Beispiel ist die chronische mukokutane Candidiasis (CMC) bei welcher chronische Infektionen mit dem Pilz *Candida* auftreten oder wiederkehren. Verschiedene Arbeiten belegen eine gestörte Dephosphorylierung von STAT1 aufgrund von Mutationen in der CC-Domäne oder der DBD, welche zu einer übermäßigen STAT1-Phosphorylierung und missgeleiteten T-Zell Entwicklung führen [Liu, 2011, Smeekens, 2011, van de Veerdonk, 2011, Takezaki, 2012].

Der von uns aufgeklärte Zusammenhang zwischen STAT1-Acetylierung und beschleunigter Dephosphorylierung könnte für die Therapie solcher Erkrankungen von Interesse sein. Daraus ergeben sich vielversprechende Behandlungsansätze für die Verwendung von HDACi.

6. Zusammenfassung

Posttranslationale Modifikationen können die Funktionen von Proteinen entscheidend verändern. Neben der Phosphorylierung hat die Acetylierung von STAT-Proteinen in den letzten Jahren zunehmend an Bedeutung gewonnen. Unsere Arbeitsgruppe hat einen grundlegenden Beitrag zur Aufklärung der Acetylierung von STAT1 geleistet.

STAT1 ist in die Regulation des Immunsystems eingebunden und steuert außerdem inflammatorische und antiproliferative Prozesse. Es gilt als Tumorsuppressor und vermittelt pro-apoptotische Vorgänge. Eine Acetylierung von STAT1 wird beispielsweise durch Interferone (IFNs) und Histondeacetylase-inhibitoren (HDACi) hervorgerufen und kontrolliert die STAT1-Aktivität. IFNs bewirken zunächst eine Aktivierung von STAT1 durch Tyrosinphosphorylierung. Bei langanhaltender Stimulation wird jedoch eine Acetylierung eingeleitet. Diese Acetylierung hat einen hemmenden Effekt, der durch die T-Zell-Protein-Tyrosin-Phosphatase (TCP45) vermittelt wird und für die Verhinderung von überaktivem STAT1 unverzichtbar ist.

In der vorliegenden Dissertation konnte der hemmende Einfluss verschiedener HDACi auf die IFNγ-induzierte STAT1-Phosphorylierung beschrieben werden. Es war erstmalig möglich diesen Effekt in primären humanen Zellen zu beobachten. Durch FRET-Analysen konnte eine verstärkte Interaktion zwischen TCP45 und Acetyl-STAT1 nachgewiesen werden.

Weiterhin konnte in dieser Arbeit gezeigt werden, dass die funktionelle Konsequenz der STAT1-Acetylierung im Zusammenhang mit dem stimulierenden IFN zu sehen ist. Acetylierung-simulierendes $STAT1^{QQ}$ kann durch chemische Hemmung oder genetischen *knock down* der TCP45 und anschließender IFNα- sowie IFNγ-Stimulation phosphoryliert werden. Dennoch ist nur im Fall von IFNα und nicht nach IFNγ-Behandlung die Transkriptionsfähigkeit von $STAT1^{QQ}$ wiederhergestellt. IFNα bewirkt die Aktivierung von verschiedenen STATs wie beispielsweise STAT1, STAT2 und STAT3. Dementsprechend ist die Bildung von Heterodimeren möglich, wo ein funktioneller Heterodimerpartner das inaktive $STAT1^{QQ}$ im „Huckepack“-Mechanismus an die DNA rekrutieren kann. IFNγ hingegen aktiviert ausschließlich STAT1 wodurch lediglich Homodimere formiert werden und $STAT1^{QQ}$ inaktiv bleibt.

STAT1 weist mit STAT3 eine große strukturelle Ähnlichkeit auf. Dennoch wird STAT1 durch Acetylierung negativ beeinflusst, STAT3 hingegen wird aktiviert. Interessanterweise sind die entsprechenden acetylierbaren Lysinreste von STAT1 in STAT3 Arginine, welche nicht acetyliert werden können. Wir mutierten die Arginine zu Lysinen beziehungsweise Glutaminen und übertrugen so das STAT1-Acetylierungsmodul auf STAT3. Die STAT3-Doppellysinmutante ($STAT3^{KK}$) und eine Glutaminmutante von STAT3 ($STAT3^{R414Q}$) zeigten

eine verringerte Phosphorylierung und eine beeinträchtigte Transkriptionsregulation. Für $STAT3^{R414Q}$ konnte eine verstärkte TCP45-abhängige, für $STAT3^{KK}$ sogar eine HDACi-vermittelte Inaktivierung nachgewiesen werden. Demnach ist die inhibierende Eigenschaft des STAT1-Acetylierungsmoduls auf STAT3 übertragbar.

Wir untersuchten weiterhin eine N-terminale Mutante von STAT1 ($STAT1^{AA}$), welche dauerhaft phosphoryliert ist und einen monomeren Charakter aufweist. Es gelang Acetylierung simulierende Glutamine in $STAT1^{AA}$ einzuführen, die eine Phosphatase-abhängige Inaktivierung erlauben.

Sowohl die verwendeten STAT3-Mutanten als auch die N-terminalen STAT1-Mutanten belegen eine inhibierende Wirkung des STAT1-Acetylierungsmoduls. Zusätzlich bestätigen sie durch die Übertragbarkeit des Moduls das Prinzip der STAT1-Acetylierung und geben Hinweise auf einen evolutionären Regulationsmechanismus zwischen STAT1 und STAT3, der auf Acetylierung beruht.

Durch ein Wechselspiel von Phosphorylierung und Acetylierung ist eine Feinabstimmung der STAT1-Aktivität möglich. Fehlreguliertes STAT1 ist Ursache vieler Autoimmunerkrankungen und beteiligt an chronischer Entzündung. HDACi können den Anteil an acetyliertem und folglich inaktivem STAT1 erhöhen und sind deshalb vielversprechende Wirkstoffe für die Therapie der genannten Erkrankungen.

7. Summary

Posttranslational modifications could change the functions of proteins completely. Besides phosphorylation, acetylation of STAT proteins gained increasing attention during the last years. Our lab could contribute fundamental data regarding the acetylation of STAT1.

STAT1 is involved in the regulation of the immune system and it mediates inflammatory and antiproliferative processes. Furthermore, it is known as a tumor suppressor as well. An acetylation of STAT1 could be caused by interferons (IFNs) or histondeacetylase inhibitors (HDACi) and controls the STAT1 activity. IFNs initially provoke phosphorylation, which induces acetylation of STAT1 during permanent stimulation. Acetylation mediates a T cell protein tyrosine phosphatase (TCP45)-dependent inhibitory effect on STAT1 signaling, preventing persistently activated STAT1.

This Ph. D. thesis reveals a negative impact of different HDACi on IFNγ-induced phosphorylation of STAT1. This effect could be observed in primary human cells for the first time. Using FRET analysis we detected an increased interaction between TCP45 and acetylated STAT1.

Furthermore, this thesis suggests that the functional outcome of STAT acetylation is dependent on the type of interferon, which is used for stimulation. The acetylation mimicking mutant $STAT1^{QQ}$ could be phosphorylated upon chemical inhibition or genetic *knock down* of TCP45 following incubation with IFNα or IFNγ. However, only IFNα but not IFNγ stimulation enabled transcriptional activity of $STAT1^{QQ}$. IFNα induces activation of different STATs, for instance STAT1, STAT2 and STAT3. Therefore, the formation of heterodimers is possible and an intact heterodimer partner could recruit the inactive $STAT1^{QQ}$ in a piggy-back mechanism to the DNA. In contrast, IFNγ activates exclusively STAT1 and hence generates $STAT1^{QQ}$/$STAT1^{QQ}$ homodimers which are inactive.

STAT1 and STAT3 show a high structural homology. However, acetylation affects STAT1 negatively whereas STAT3 is activated by this modification. Interestingly, STAT3 contains two arginine moieties, which are not acetylatable, corresponding to two lysine moieties in STAT1. We changed these arginines to lysine or glutamine residues and thereby transferred the STAT1 acetylation module to STAT3. A double lysine mutant ($STAT3^{KK}$) and a glutamine mutant ($STAT3^{R414Q}$) revealed a reduced phosphorylation and impaired transcriptional activity. $STAT3^{R414Q}$ showed an increased TCP45-dependent inactivation and $STAT3^{KK}$ even an HDACi-dependent inactivation. These results indicate that the inhibiting character of the STAT1 acetylation module could be transferred to STAT3 as well.

Furthermore, we investigated N-terminal mutants of STAT1 ($STAT1^{AA}$), which show prolonged phosphorylation and possess a monomeric character. We were able to introduce

acetylation-mimicking glutamines to $STAT1^{AA}$, leading to phosphatase-dependent inactivation.

Both, the STAT3 mutants as well as the N-terminal STAT1 mutants confirmed the negative impact of the STAT1 acetylation module on signaling. Additionally, these results show that the acetylation module is transferable and hence confirm the principle of STAT1 acetylation. Besides, we suggest an evolutionary developed mechanism for STAT1 and STAT3 regulation by acetylation. A phosphorylation/acetylation switch enables fine tuning of STAT1 activity and thus prevents overactive STAT1. Persistently activated STAT1 promotes autoimmunity diseases and is involved in chronic inflammation. Since HDACi enhance the pool of inactive acetyl-STAT1, they are promising candidates for therapy of these diseases.

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9. Eigenständigkeitserklärung

Hiermit versichere ich, dass ich die Promotionsordnung eingehalten habe. Die vorliegende Dissertation wurde von mir selbst angefertigt. Ich habe keine Textabschnitte eines Dritten ohne Kenntlichmachung des Zitats übernommen. Weiterhin habe ich alle von mir eingesetzten Hilfsmittel, persönliche Mitteilungen und Quellen angegeben.

Jena, den ……..……. ……..

Torsten Ginter

10. Tabellarischer Lebenslauf

PERSÖNLICHE DATEN

Name:	Torsten Ginter
Geburtstag:	07.04.1982
Geburtsort:	Hoyerswerda
Anschrift:	Katharinenstraße 24 07743 Jena
Familienstand:	verheiratet, eine Tochter

AUSBILDUNG

02/2009 - 03/2013	wissenschaftlicher Mitarbeiter mit dem Ziel einer Promotion zum Thema: „Untersuchung der funktionellen Bedeutung der Acetylierung von STAT1 und STAT3“ Center for Molecular Biomedicine (CMB) Institut für Biochemie und Biophysik Friedrich-Schiller-Universität Jena PD Dr. O.H. Krämer
10/2007 – 12/2008	Diplomarbeit mit dem Thema: „Vergleichende Untersuchung der STAT3-/STAT1-Interaktion im Interferon α-/Interleukin 6-Signalweg“ Center for Molecular Biomedicine (CMB) Institut für Biochemie und Biophysik Friedrich-Schiller-Universität Jena Prof. Dr. T. Heinzel
10/2003 – 10/2007	Friedrich Schiller Universität Jena Studium der Biochemie/ Molekularbiologie
08/2001 – 06/2003	Staatliche Gewerbeschule Hamburg Ausbildung zum Biologisch-technischen-Assistenten
08/1995 – 06/2001	Spreeland Gymnasium Cottbus Abitur

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(Torsten Ginter)

PUBLIKATIONEN

2012 Histone deacetylase inhibitors block IFNγ-induced STAT1 phosphorylation.

Ginter T, Bier C, Knauer SK, Sughra K, Hildebrand D, Münz T, Liebe T, Heller R, Henke A, Stauber RH, Reichardt W, Schmid JA, Kubatzky KF, Heinzel T, Krämer OH.
Cell Signal. 2012 Jul;24(7):1453-60. Epub 2012 Mar 7.

Acetylation modulates the STAT signaling code.

Wieczorek M, **Ginter T**, Brand P, Heinzel T, Krämer OH.
Cytokine Growth Factor Rev. 2012 Dec;23(6):293-305. Epub 2012 Jul 13.

2013 Acetylation of Endogenous STAT Proteins.

Ginter T, Heinzel T, Krämer OH.
Methods Mol Biol. 2013;967:167-78.

Regulation of STAT3 and STAT1 by acetylation-phosphorylation cassettes

Ginter, T.; Kosan, C.; Bier, C.; Stauber, R.H.; Henke, A.; Heinzel, T.; and Krämer, O.H
Manuskript in Vorbereitung

Weitere Publikationen, die nicht in diese Dissertation eingebunden sind:

2012 A combination of a ribonucleotide reductase inhibitor and histone deacetylase inhibitors downregulates EGFR and triggers BIM-dependent apoptosis in head and neck cancer.

Stauber RH, Knauer SK, Habtemichael N, Bier C, Unruhe B, Weisheit S, Spange S, Nonnenmacher F, Fetz V, **Ginter T**, Reichardt S, Liebmann C, Schneider G, Krämer OH.
Oncotarget. 2012 Jan;3(1):31-43.

2013 Interferon alpha-armed nanoparticles trigger rapid and sustained STAT1-dependent anti-viral cellular responses

Pollok S, **Ginter T**, Günzel K, Pieper J, Henke A, Stauber R, Reichardt W, and Krämer OH
Cellular Signalling (akzeptiert 10.01.2013)

The ubiquitylating enzymes UBCH8 and SIAH modulate cancer-relevant Janus kinase signaling

Müller, S., Chen, Y., Buchwald, M., Schäfer, C., **Ginter, T.**, Petersen, I., Heinzel, T., and Krämer, O.H.
Manuskript in Vorbereitung

POSTERPRÄSENTATIONEN

2011 Acetylation impairs activation of STAT1 homodimers

Ginter T, Heinzel T., Reichardt W., and Krämer O.H.
5th Mildred Scheel Cancer Conference, 13.-15. Juli 2011, Königswinter (bei Bonn)

11. Angaben zum Eigenanteil

Manuskript 1

Titel: Histone deacetylase inhibitors block IFNγ-induced STAT1 phosphorylation.

Autoren: Torsten Ginter, Carolin Bier, Shirley K. Knauer, Kalsoom Sughra, Dagmar Hildebrand, Tobias Münz, Theresa Liebe, Regine Heller, Andreas Henke, Roland H. Stauber, Werner Reichardt, Johannes A. Schmid, Katharina F. Kubatzky, Thorsten Heinzel, Oliver H. Krämer,

Beitrag zum Manuskript:

Alle Experimente und Arbeiten bezüglich des Manuskripts wurden von mir durchgeführt. Ausnahmen bilden die folgenden Punkte:

Dagmar Hildebrand und Katharina Kubatzky lieferten die Daten für Abbildung 1C. Die FRET-Analysen zu 2D und 3C wurden von Kalsoom Sughra und Johannes A. Schmid durchgeführt. Die Immunfluoreszenz-Daten aus Abbildung 3D, S5 und S7 stammen von Carolin Bier und Shirley Knauer. Der EMSA zu Abbildung 6E wurde durch Tobias Münz gemacht. Zusammen mit Heike Urban wurden die CPE-Assays für 6F durchgeführt. Die FACS-Daten aus S3 wurden durch Claudia Schäfer generiert. Abbildung S4 liegen Daten von Oliver Krämer zugrunde. Oliver Krämer und Thorsten Heinzel haben die Erstellung des Manuskripts betreut und geleitet. Alle restlichen Autoren haben zum Erfolg des Manuskripts durch konstruktive Hinweise und Diskussionen beigetragen.

Manuskript 2

Titel: Acetylation modulates the STAT signaling code

Autoren: Martin Wieczorek, Torsten Ginter, Peter Brand, Thorsten Heinzel, Oliver H. Krämer

Beitrag zum Manuskript:

Sowohl Martin Wieczorek, Peter Brand als auch ich haben maßgeblichen Anteil an der Planung und Ausführung des Manuskripts gehabt. Martin Wieczorek erstellte Abbildung 1 und Abbildung 4. Abbildung 2 wurde von Peter Brand gestaltet. Der Schwerpunkt von Martin Wieczorek lag auf dem Teil, der sich mit STAT3 und SIRT1 sowie mit der Regulation von immunologischen Funktionen durch STAT3-Acetylierung befasst. Zusätzlich trug er entscheidend zu dem Abschnitt bei, der STAT5-Acetylierung behandelt. Ich steuerte Tabelle 1, Tabelle 2 sowie Abbildung 3 bei. Besonderen Anteil hatte ich an den Teilstücken, die sich mit STAT1- und STAT3-Acetylierung beschäftigen. Oliver Krämer und Thorsten Heinzel haben den Entstehungsprozess des Manuskripts konstruktiv betreut und geleitet.

Manuskript 3

Titel des Buchkapitels: Acetylation of Endogenous STAT Proteins

Autoren: Torsten Ginter, Thorsten Heinzel, Oliver H. Krämer

Beitrag zum Manuskript: Die experimentellen Daten für Abbildung 1 und 2 wurden von Oliver Krämer zur Verfügung gestellt. Alle anderen Abbildungen und Arbeiten wurden von mir ausgeführt. Oliver Krämer und Thorsten Heinzel haben die Fertigstellung des Manuskripts betreut und mit konstruktiven Vorschlägen zum Gelingen beigetragen.

Manuskript 4

Titel: Regulation of STAT3 and STAT1 by acetylation-phosphorylation cassettes

Autoren: Torsten Ginter, Christian Kosan, Carolin Bier, Roland H. Stauber, Andreas Henke, Thorsten Heinzel, and Oliver H. Krämer

Beitrag zum Manuskript: Die Immunfluoreszenz-Mikroskopie für Abbildung 1C wurde von Carolin Bier durchgeführt. Theresa Liebe steuerte die Experimente für Abbildung 4B bei. Zur Abbildung 5B hat Heike Urban mit Infektion und kolorimetrischer Auswertung der Zellen beigetragen. Der Rest der Experimente stammt von mir. Der Text und die Abbildungen wurden durch mich in Abstimmung mit Oliver Krämer und Christian Kosan erstellt. Die übrigen Co-Autoren haben hilfreich durch konstruktive Diskussionen zur Erstellung des Manuskripts beigetragen.

Die Arbeitsanteile aller beteiligten Autoren wurden in Inhalt und Umfang richtig ausgewiesen.

.....

(Betreuer: PD Dr. Oliver H. Krämer)

12. Danksagung

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